MECHANISMS FOR MYOCARDIAL OXIDATIVE STRESS IN OBESITY

By

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This dissertation is dedicated to my husband, Kevin, and my "little buddy" Ian.
You are my inspiration and the lights in my life.

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More than anything, I wanted to leave the Center for Exercise Science with the sense that I had contributed somehow toward making where I studied and worked a better place for my having been there. I hope that the spirit and forays of this project into a different area have helped to do just that. I can be sure, however, that I have become a better person for having been *here*. I owe this is great part to my friend and mentor, Dr. Scott Powers. There was no better education than the understanding, trust, and faith that came with his friendship during my stay here. He has helped me in many ways evolve into who I have become, and he has clarified the direction in which my efforts have led me. I can only hope that this is just the beginning of a lifelong friendship. As a person and scientist, he is a hard example to follow. I will do my best to represent him well in my career.

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Obesity is associated with increased myocardial oxidative stress, yet the mechanism(s) responsible for this damage are unknown. We hypothesized that elevated heart work, an increased rate of superoxide ($O_2^{\cdot-}$) production, increased myocardial lipid content, and insufficient antioxidant defenses contribute to oxidative stress in obesity. To test this hypothesis, Zucker rats (7 weeks old) were fed experimental diets for 9 weeks to promote obesity by high-fat intake or lack of expression of the leptin receptor. Lean control rats (CON, Fa/?) were fed either a control diet (10% fat) or a high-fat diet (FAT, 45% fat), while obese rats (OB, fa/fa) were fed the control diet. Oxidative stress was assessed by measurement of hydroperoxides (PEROX) and thiobarbituric reactive acid substances (TBARS).

Compared to CON, the FAT and OB had similar elevations in PEROX and TBARS (+21% and +33%, respectively, $p<0.05$). Small but significant differences ($p<0.05$) in resting heart work (heart rate X systolic blood pressure) existed between the FAT and OB compared to CON. Activities of antioxidant enzymes CuZn-superoxide dismutase and catalase and endogenous glutathione levels were elevated (32%, 15%, and 18%, respectively, $p<0.05$) in OB compared to CON. Myocardial lipid content was increased similarly among all FAT and OB animals ($p<0.05$) compared to CON. The rate of $O_2^{\cdot-}$ formation by isolated papillary muscles in vitro did not differ among the experimental groups ($p<0.05$). Regression analysis revealed that the largest contributor to oxidative damage was myocardial lipid content ($R^2=0.76$, $p<0.05$). These data indicate that myocardial oxidative injury is not closely linked with elevated heart work, insufficient antioxidant defenses or a greater rate of $O_2^{\cdot-}$ production. In contrast, myocardial lipid content is a key contributor to obesity-related myocardial oxidative stress.

CHAPTER 1
INTRODUCTION

Obesity is a serious clinical disorder affecting millions of Americans (33%), and the incidence is steadily increasing per year (7, 26). An alarming trend is the increase in the percentage of adolescents and young adults who are becoming obese (estimates ranging from 35 to 40%), with an ever-rising percentage of obese youths becoming severely obese (51). Obesity is an independent risk factor for cardiovascular disease and increased mortality (52) The underlying mechanisms for this increased morbidity and mortality are unknown. Information is scarce regarding the myocardial alterations that occur at the cellular level that make the obese individual more prone to myocardial injury or irreversible damage leading to death. Hence, research in this area is clearly warranted

A preliminary biochemical investigation in our laboratory indicates that obesity is associated with increased myocardial lipid peroxidation and susceptibility to oxidative damage *in vitro* (111). The potential consequences of oxidative damage can be severe, studies examining oxidative stress and the myocardium have shown that cellular membrane integrity can be lost (97, 114), and lipids and proteins are transiently or irreversibly altered, resulting in myocardial contractile dysfunction (40). These factors can ultimately lead to cardiac arrhythmias, poor contractility, infarction, cardiac failure, or sudden death (7)

Genetics and environment have both been identified as major contributors in the etiology of obesity (11). The individual effects of these factors on the predisposition to oxidative stress in the myocardium is unknown. The current animal models used in the study of the systemic effects of obesity include the widely accepted genetically inbred fatty Zucker rat (fa/fa) and the overfed, overweight rat. Although both models increase myocardial work (e.g., elevated systolic blood pressure), the acquired and genetic obesity models have different influences on myocardial work and possibly the level or type of oxidative stress. The significance of this issue is such that in human obesity, there are both heritable and environmental factors that are involved in the pathogenesis of obesity and its association with cardiovascular disease (11). Recent evidence suggests that oxidative stress is involved in the cellular damage incurred by cardiovascular disorders such as coronary artery disease, hypertension, atherosclerosis, and vasospastic angina (27). Considering that obesity (a) is often accompanied by these cardiovascular disorders, and (b) is associated with myocardial oxidative stress, it is important to examine oxidative cellular injury in both genetic and acquired obesity models.

There are several potential mechanisms to explain the increase in myocardial lipid peroxidation associated with obesity: (1) increased myocardial work and oxygen flux through the mitochondrial respiratory chain (97); (2) a decreased myocardial antioxidant defense (23,24); (3) increased fat deposition within myocardial tissue (63); and (4) increased rates of radical formation (12). Although there is indirect evidence to support

that all these mechanisms contribute to myocardial oxidative damage in the obese, direct evidence is lacking. In addition, it in unclear how each of these factors are differentially influenced by the fa/fa genotype and diet. Therefore, this investigation will examine the relationships between oxidative stress and the cellular characteristics of the myocardium from animals of two obesity models: high-fat fed animals and animals possessing the leptin receptor defect (fa/fa). We attempted to determine the influence of the leptin receptor defect (fa/fa) and high-fat feeding on myocardial lipid peroxidation.

Specific Aims

Obesity is associated with increased myocardial lipid oxidative damage in the obese fatty Zucker rat (111). It is unknown whether these obese animals and lean animals that are fed a high-fat diet are at the same risk for myocardial lipid peroxidation. Several lines of evidence suggest that there are many potential mechanisms that could promote lipid peroxidation in the myocardium in either the fa/fa rat or the high-fat fed animal. This investigation will compare the cellular antioxidant characteristics and *in vitro* responses of myocardial tissue from genetically lean (Fa/?), genetically obese (fa/fa), and high-fat fed rats (Fa/?). Therefore, the specific aims of this project are as follows:

Specific Aim 1: To determine if high-fat fed rats (Fa/?, 44.9% dietary fat) and the obese rat (fa/fa, 10% dietary fat) experience the same levels of oxidative injury (i.e., lipid peroxidation) in the myocardium

Hypothesis 1: We hypothesize that the high-fat fed and obese animals (fa/fa) will have similar levels of lipid peroxidation. Further, we hypothesize further that the degree of lipid peroxidation will be independent of the fa/fa genotype and dependent upon the degree of adiposity.

Specific Aim 2: To systematically examine several factors which could contribute to elevated myocardial oxidative stress (i.e , lipid peroxidation) in obesity. These include: (1) increased heart work (rate pressure product) due to obesity; (2) insufficient intracellular primary antioxidants such as antioxidant enzymes and glutathione; (3) increased fat deposition within myocardial tissue; and (4) increased rate of radical formation (superoxide) by isolated papillary muscles form the heart.

We will test the following hypotheses:

Hypothesis 2a: Obese animals will have a higher double product (i.e., heart rate X systolic blood pressure) compared to lean animals.

Hypothesis 2b: Glutathione levels and antioxidant enzyme activities will be reduced in hearts of obese animals compared to lean animals.

Hypothesis 2c: Myocardial tissue obtained from the left ventricles of obese animals will contain more lipid compared to myocardial tissue obtained by lean animals.

Hypothesis 2d: Contracting papillary muscles from obese animals will produce superoxide anions at a greater rate compared to lean animals.

Hypothesis Justification

We hypothesize that the degree of myocardial lipid peroxidation will not differ between high-fat fed rats (Fa/?) and the obese fa/fa rats. The factors which can increase oxidative stress on the heart are similar between both of these animal models. Specifically, obesity-induced hypertension has been documented in both overfed animals fed high-fat diets (14, 54, 55) and in obese Zucker rats (18). Hypertension forces the myocardium to work at greater workloads independent of genotype (14, 61). The myocardial rate pressure product and oxygen uptake increase in obesity. These processes can lead to excessive $O_2^{\cdot}$ production. In addition, the presence of excessive fat deposition within the myocardium in genetically obese or overfed, overweight animals serves as an enlarged target for lipid peroxidation (63). These factors appear to be independent of genetics. Hence, we speculate that lipid peroxidation levels will be the same in both groups of obese animals.

Our second series of hypotheses relate to factors contributing to increased myocardial lipid peroxidation in obese animals. First, hypothesis 2a states that obese animals will have a higher double product compared to lean animals. As previously mentioned, obesity places a substantial mechanical load on the heart which increases myocardial oxygen consumption, as evidenced by the increased rate pressure product (2, 14). Second, hypothesis 2b states that glutathione and antioxidant enzyme levels will be

reduced in hearts of obese animals compared to lean animals. Obesity in humans and animals is associated with lowered serum or tissue vitamin E, β-carotene, and/or tissue glutathione (19, 24, 79, 91), and some reports indicate reduced antioxidant enzyme activities. Hypothesis 3b postulates that the left ventricles of obese (fa/fa) animals will contain more lipid compared to ventricular tissue of lean animals. Increased deposition of (polyunsaturated or saturated) fats within tissues is common to obesity and increases the risk for lipid peroxidation by increasing the oxidation target number (7, 62, 63, 78). Lastly, hypothesis 4b states that contracting papillary muscles from obese animals will produce superoxide anions at a greater rate compared to lean animals. It has been suggested that there is a greater mitochondrial lipid oxidation rate in tissue of obese animals (12) providing indirect evidence for the notion that the elevated oxygen flux through the mitochondria may be a primary source of ROS production within myocytes from obese animals (12).

Significance

Obesity is an increasingly prevalent metabolic disorder affecting not only the U.S. population but also that of the developing world (26). It is associated with many comorbidities and it complicates health conditions in patients with various cancers, diabetes, and cardiovascular disease (7, 25). Obesity is linked with a high morbidity and mortality rate, particularly among cardiovascular patients (52). More frequent have the

incidences of fatal and non-fatal heart disease, arrhythmias and sudden death become in obese persons during recent years (26). The economic burden that obesity and its related disorders place on the U.S. health care system is enormous The direct cost of obesity alone was recently estimated at $45.8 billion (52). However, this estimate is low due to the fact that many of the disorders, such as heart disease, that arise from obesity are classified as illnesses separate from obesity; an additional $ 23 billion are used to treat these disorders that are associated with obesity.

The prevalence of obesity in the adult population is rapidly increasing by 0.6% per year for men and 1% per year for women (currently, ~33 % of the adult population is severely obese)(7, 26). More alarming is the fact that the percentage of children that are becoming obese is rising rapidly (51). These statistics suggest that obesity is a disorder that will continue to affect our nation's population and drain our health care resources The U.S. will continue to suffer economically as a result of this disease Hence, it is essential that we expand our knowledge about the obesity syndrome.

CHAPTER 2
LITERATURE REVIEW

Introduction

Obesity is a prevalent disorder in the U.S. population and is also becoming an epidemic in European countries, Canada and the third world (7). According to the recent phase of the National Health and Nutrition Examination Survey (NHANES III, 1988-1991), approximately 33% of the U.S. population is obese, 8% more than when the last phase of NHANES II was completed (1976-1980) (60).

Obesity is defined as the accumulation of excess fat, such that the body mass index (BMI, mass/height^2) is greater than 30 kg/m^2 (52). This overfatness is associated with a number of comorbidities, including many forms of heart disease. Fat distribution also represents risk for heart disease; deposition of fat in the abdomen indicates a greater risk for coronary heart disease mortality (7).

Obesity can develop as a consequence of environment, such that overeating or consumption of a high-fat diet induces fat accretion that may exacerbate weight gain. Genetics can significantly contribute to 30-70% of the cases of obesity by influencing fat accretion throughout life and passing on genes that predispose the offspring to obesity-related complications (10, 52). While it is well established that obesity is associated is an increased risk of heart disease, recent evidence also indicates that obesity is also associated with an increase in oxidative damage to the myocardium (111). The purpose of this review

is to discuss possible physiological and biochemical links between obesity and myocardial oxidative injury.

The Obesity Syndrome

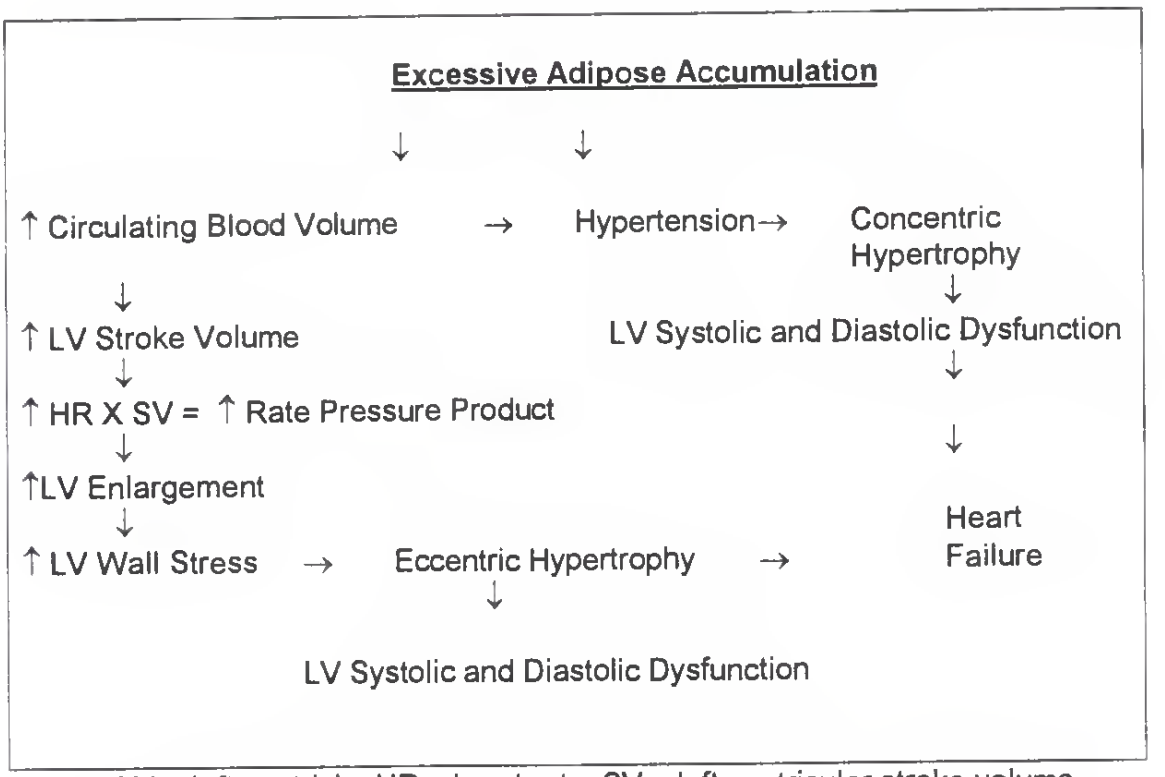
Obesity is characterized by a complex pathophysiology that can impose potentially harmful consequences on the cardiovascular system. Obesity is often accompanied by increased plasma volume and hypertension, poor glycemic control, hyperlipidemia and increased adrenergic drive with reduced adrenergic sensitivity at the tissue level (15, 88). Further, the myocardium is often hypertrophied and may have fatty infiltration (2).

Obesity can have deleterious cardiovascular effects, but could also initiate several cellular pathways that may promote myocardial oxidative damage. Specifically, the workload on the heart is increased by obesity (15); this would increase the oxygen flux through the mitochondrial respiratory chain (12). Further, the myocardial antioxidant defense may be insufficient to protect against damage by reactive oxygen species (ROS) (23, 24). Finally, there may be fatty infiltration and increased polyunsaturated fat deposition within myocardial tissue that increases the risk for oxidation by ROS (63).

Obesity and Myocardial Overload

There are several ways in which obesity contributes to an excessive workload on the heart. Figure 1 summarizes the potential mechanisms involved in obesity-induced

myocardial overload. Excessive weight gain is associated with an increased plasma volume and subsequent blood volume expansion (4). In addition, the hypertension so often associated with obesity can be induced by insulin-mediated mechanisms. Hyperinsulinemia causes sodium and water retention by acting directly on the renal tubules and on the renin-angiotensin-aldosterone system, and it can also promote arterial smooth muscle proliferation (88), all of which increase blood pressure and end diastolic volume. Increased volume enhances diastolic filling and places a stretch overload on the heart (increased preload). The wall stress in the ventricles increases. According to *LaPlace's law* (wall stress = pressure X radius/ 2 X wall thickness), the bigger the left ventricle and/or the greater the pressure developed by the left ventricle, the greater the wall stress. An increase in wall stress increases myocardial O_2 uptake as more ATP must be used to generate greater tension to contract against this wall stress (80). In response to this wall stress, eccentric and/or concentric cardiac hypertrophy will occur. Although gross mechanical function may appear normal, there are several subtle mechanical alterations that affect cardiac performance. Specifically, systolic function is compromised such that the rate of shortening velocity is reduced (80). Also, diastolic dysfunction occurs. Relaxation time is delayed and peak filling rates are reduced (2). In some specific cases, vascular resistance may be reduced to counteract the increased blood volume. This may override the renin-angiotensin effects. Diastolic filling is compromised and stroke volume is reduced.



where LV = left ventricle, HR = heart rate, SV = left ventricular stroke volume

Figure 1. A schematic representation of the pathogenesis of heart complications induced by obesity.

Therefore, the heart rate must increase in turn to maintain cardiac output (CO) (2, 80). In all scenarios, the heart is working at higher work rates in obesity

Hypertension is present in approximately 60% of obese individuals, with 10% of those cases being classified as severe (94). The myocardium of hypertensive obese individuals works against a greater systemic resistance created by elevated blood pressure (increased afterload). Specifically, blood pressure is increased on average 6 mmHg systolic and 4 mmHg diastolic for each 10% gain in body fat, with a greater effect observed in those genetically susceptible to obesity (52).

Systolic dysfunction may manifest as depression of left ventricular peak rate of contractility. Furthermore, obesity-induced hypertrophied hearts are susceptible to potentially fatal arrhythmias, or heart failure (14).

In summary, the neuroendocrine and mechanical alterations that occur with obesity are associated with an increase in the stress placed on the heart. Blood pressure and heart rate are typically elevated, and preload and afterload are increased. The myocardium hypertrophies to counteract this overload stress. Alterations in heart performance include depressed rate of contractility, relaxation, and manifestation of arrhythmias or failure.

Myocardial Production of Reactive Oxygen Species

A radical is a molecule or a molecular fragment containing an unpaired electron. In general, approximately 2-5% of the oxygen consumed used during oxidative metabolism is

transformed to radicals or other reactive oxygen species (ROS) (48, 49). ROS are considered essential in cellular homeostasis and when present in small amounts have been shown to enhance contractile processes (86). ROS are often scavenged by naturally occurring protective antioxidant defenses such as enzymes, vitamins and other molecules within the myocyte so that an antioxidant-prooxidant balance is achieved. Under pathological conditions or conditions in which electron flux through the electron transport chain increases (such as exercise or increased contractile activity), ROS production increases and causes a prooxidant state within the cell (114). When the cellular antioxidant defenses are overpowered, this causes major disruptions to muscle contractile function, cellular homeostasis and subsequent damage termed "oxidative stress" (49, 86). This section will highlight the potential sources of ROS in cells and the types of molecules generated.

Major ROS and Sources of ROS

The major ROS include superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH.); other contributing ROS are the nitrogen containing species such as peroxynitrite (ONOOH) and nitric oxide ($NO^{\cdot}$). Molecular oxygen itself is a diradical, though not highly reactive. The major potential sources of myocardial ROS in obesity are the mitochondrial respiratory chain, xanthine oxidase activity, the neutrophil oxidative burst, nitric oxide synthesis, catecholamine oxidation (49, 112). In the healthy

myocardium, a small fraction of the electrons flowing through the electron transport chain leak from the reaction paths and collide with O_2 to form superoxide radicals (48). This anion is produced at greater rates under conditions that stress the respiratory chain, including greater myocyte contraction rates (49). Superoxide can react with another O_2^- radical and two hydrogens to form H_2O_2 ; direct dismutation of O_2^- via superoxide dismutase can produce H_2O_2 in the mitochondrion (Asayama et al. 1991, Girotti 1998). Though H_2O_2 is not a radical, it is a reactive oxygen specie and can cause serious cellular damage if it accumulates (48). Homolytic fission of the O-O bond in H_2O_2 produces two hydroxyl radicals (OH), and can be achieved by an iron salt and H_2O_2 by Fenton chemistry. The OH molecules react with an extremely high rate constant especially with nearby biological membranes (34, 67). Therefore, OH^- reacts very close to its point of formation in a phenomenon known as "site specific" reactivity (34).

Mainly located in the vessel walls of most tissues, xanthine dehydrogenase catalyzes the oxidation of hypoxanthine to xanthine, and xanthine to uric acid. Xanthine oxidase uses O_2 as the electron acceptor and produces O_2^- while catalyzing the oxidation of hypoxanthine to uric acid (114)

Another major source of ROS in cardiovascular disease is the reaction of O_2^- and NO that occurs within the milieu of neutrophilic and macrophage oxidative bursts. Upon phagocytic cell infiltration at the site of injury, these cells release a mixture of these two ROS that form the strong oxidant peroxynitrate (ONOOH). Also, activated neutrophils

release myeloperoxidase-generated hypochlorous acid (HOCl) which is a strong oxidant that can cause serious cellular damage (102, 112). It is unclear whether obesity per se initiates myocardial infiltration by neutrophils, but studies have implicated phagocytic infiltration of atherogenic plaques and ischemic/necrotic cardiac tissue as complications often associated with obesity (102).

While there are many targets for oxidative damage in the myocyte, these processes elicit significant damage to the myocyte membranes (22). Membrane damage results in alterations which could lead to ion imbalance and subsequent myocardial dysfunction (63). It is well documented that the oxidative stress resulting from events such as ischemia-reperfusion cause serious depression in myocyte function, such as inability to develop maximal force, reduction in the rate of force development, and reduced segment shortening (72). Therefore, the myocardium has several defense mechanisms to counteract these potential effects.

Myocardial Antioxidant Defense

The endogenous antioxidant defense within the myocyte includes both enzymatic and non-enzymatic components. The defense system exists as a multilevel system, such that the major protection against acute oxidative stress is provided by glutathione, primary antioxidant enzymes and dietary antioxidants (20). The secondary defense includes repair

enzymes, such as lipolytic and proteolytic enzymes, proteases and phospholipases that repair cellular damage following oxidative stress.

Enzymatic Defense

The myocardial antioxidant enzymatic defense system includes superoxide dismutase (SOD) which scavenges $O_2^{\cdot-}$, catalase (CAT) which scavenges H_2O_2 and glutathione peroxidase (GPX) which reduces lipid peroxides and neutralizes H_2O_2. These enzymes exist in differing concentrations in varying compartments within the myocyte, thus providing a strategic defense against ROS generated *in vivo*. Specifically, the manganese-dependent isoform of SOD (Mn-SOD) is found in the mitochondria, the copper/zinc-dependent SOD isoform (CuZn-SOD) is found within the cytosol (Ji 1995). GPX is found within the mitochondrial matrix and the cytosol, with most of the enzyme located within the cytosol with a ratio of ~2:1 (49). GPX is activated by H_2O_2 at lower concentrations (K_m = 1 μM) whereas the other H_2O_2 scavenger, CAT, is activated by higher concentrations of the same substrate (K_m = 1 mM). This overlap of substrate for these two enzymes appears physiologically relevant in that if the oxidative stress of H_2O_2 exceeds the capability of GPX, CAT is present to provide protection. CAT, though found throughout the cell, can be found primarily within peroxisomes. Activity of this enzyme depends upon the binding of Fe^{3+} to the active site (114).

Glutathione

Glutathione (GSH, γ-glutamylcysteinylglycine), the predominant thiol in cells, has direct antioxidant activity and is also involved in recycling other dietary antioxidants (49). For example, GSH may neutralize hydroxyl radicals and singlet oxygen by abstracting an electron and/or donating a proton, and can maintain tissue antioxidant vitamins in the reduced state (74, 114). GSH is a substrate for GPX, which removes lipid peroxides and H_2O_2. GSH is synthesized primarily by the liver and is transported to extrahepatic tissues via the circulation. GSH is imported by the tissues and its constituent amino acids are imported by membrane-bound enzymes (39, 74). Together, these enzymes and thiol compounds serve as part of the primary defense against ROS and radicals.

Dietary Antioxidants and Myocardial Protection

Exogenous antioxidants have been extensively used to reduce the injury associated with oxidative stress in muscle tissue. Antioxidants are general scavengers of ROS and radical species, and they can work alone or in combination with each other to reduce the reactivity of the radicals.

Vitamin E belongs to a class of tocopherol phenolic molecules (α-tocopherol being the most potent) and can covert $O_2^{\cdot}$, OH and peroxyl radicals to less reactive forms (17). It is lipid soluble, and its trimethylhydroquinone head serves to break the chain reaction of lipid peroxidation that occurs in cell membranes during oxidative stress (48,

71, 106). Once oxidized in this process, the vitamin E radical can be recycled once again to its native state by other antioxidants such as vitamin C and GSH. Thus, vitamin E acts synergistically with vitamin C and glutathione during periods of oxidative stress (49, 114).

Vitamin C is water soluble and can directly scavenge $O_2^{\cdot-}$, OH and peroxyl radicals in the cytosol and plasma. In addition, vitamin C can reduce the vitamin E radical back to its original state. Oxidized vitamin C can be reduced again to its native form by electron donors such as glutathione or dihydrolipoic acid. Thus, vitamin C is extremely important in the restoration of vitamin E in the lipid components of the cell, and in the scavenging of radicals in the aqueous phase. Further, it is linked with reduced protein glycosylation, a radical-generating process (21).

β-carotene is also a lipid soluble molecule found in membranes that can scavenge singlet O_2 and $O_2^{\cdot-}$. Similar to vitamin E, this molecule can provide great protection against lipid peroxidation and has a postulated role in the reduced uptake of oxidized low density lipoproteins in the cardiac endothelium. Both benefits are associated with reduced atherosclerotic disease in the heart. β-carotene appears to be most effective at low doses (114).

Alpha-lipoic acid is an endogenous thiol containing compound that is a potent antioxidant against all major ROS It is found in low concentrations in the aqueous compartments of myocytes, and is largely bound to enzyme complexes, rendering it unavailable for scavenging ROS. As an exogenous, unbound supplement, lipoic acid may

be effective in recycling vitamin C and serving as a protective thiol-containing molecule that can aid in reuction of oxidized molecules (53).

Oxidative Injury to Myocardial Tissue

Although oxidative damage can be incurred on cellular proteins and carbohydrates (114), much of the recent work has focused on the oxidative injury to cellular membrane lipids (62, 85). Damaged lipids, as will be described in the next sections, can alter cellular homeostasis, deteriorate contractile function or cause cell death by apoptosis (34, 105). Hence, this section will focus on oxidative injury to lipids.

Lipid peroxidation is a common type of damage observed in tissue following exposure to ROS. Lipid peroxidation is the destruction of polyunsaturated fats (PUFA) in membranes and is initiated when a ROS is able to abstract an allylic H^{+} atom (1 electron reduction) from a methylene group of a PUFA molecule. This forms a reactive lipid peroxyl radical, $ROO^{\cdot}$ (where R = lipid chain, $OO^{\cdot}$ denotes peroxyl group) which can react with an adjacent PUFA, triggering exacerbating rounds of free-radical mediated lipid peroxidation (28). This process initiates a cascade of lipid peroxidation that amplifies the destructive effects of the initial peroxidation insult. Alternatively, two electron reduction reactions with lipid hydroperoxides (ROOH) can lead to formation of redox-inert alcohols, a process which serves as a secondary or "reparative" level of cytoprotection.

Recent work has shown that basal levels of lipid peroxidation by-products are elevated within the myocardium and liver tissue of obese rats (fa/fa genotype) compared to lean control animals (Fa/- genotype)(57, 111). The significance of lipid peroxidation within membranes is that the membrane fluidity is decreased and permeability is increased. Furthermore, consumption of high fat diets or diets high in unsaturated fats (especially containing $\geq$ n-3 fatty acids) exacerbates the susceptibility to free radical-mediated peroxidation (78).

Several by-products of lipid peroxidation such as lipid hydroperoxides, malondialdehyde (MDA), conjugated dienes and 4-hydroxynonenal (4HNE) are measured to determine the extent of oxidative damage incurred on the tissue (38). The most common measures used to determine the degree of oxidative injury in lipids are MDA and lipid hydroperoxides (47).

Lipid Hydroperoxides and Malondialdehyde

An outline of the postulated pathways by which hydroperoxides and MDA are produced is shown in Figure 2. The importance of measuring these two by-products is that each by-product occurs at different places within the pathway. Decomposition of PUFA by O_2 directly results in primary hydroperoxide products. Further breakdown of hydroperoxide products generates endoperoxide radicals. These radicals, when exposed to heat or acids, produce secondary MDA products (47). Because of considerable

disagreement within the literature with regard to the "optimal" lipid peroxidation measure and the inability to specifically detect from where the MDA was derived, it is recommended at least two different measures are used. Therefore, we will measure both MDA and lipid hydroperoxides to evaluate the level of lipid peroxidation in the myocardium of both lean and obese animals.

Oxidative Damage to Membranes

Lipid peroxidation reduces membrane integrity (97). A loss of membrane integrity in cardiac myocytes can lead to arrhythmias, myocyte contractile dysfunction and cell death (105). In addition, changes in membrane lipid content and permeability can alter enzymatic membrane processes (i.e., ATPase activity)(46).

The impact of peroxidative processes is enhanced when dietary consumption of PUFA increases; reports using overfeeding models indicate that cell membrane composition of liver and aortic tissue generally reflects dietary consumption of specific lipid groups, though not absolutely (46, 59, 62, 107). A consistent finding is that peroxidative injury increases as a function of dietary consumption of PUFA (46, 78, 107). The lipid radicals that form as a result of peroxidation are believed to interfere with essential membrane function of protein channels embedded within the membrane and maintenance of ion gradients between cellular compartments (97). Lipid peroxidation of

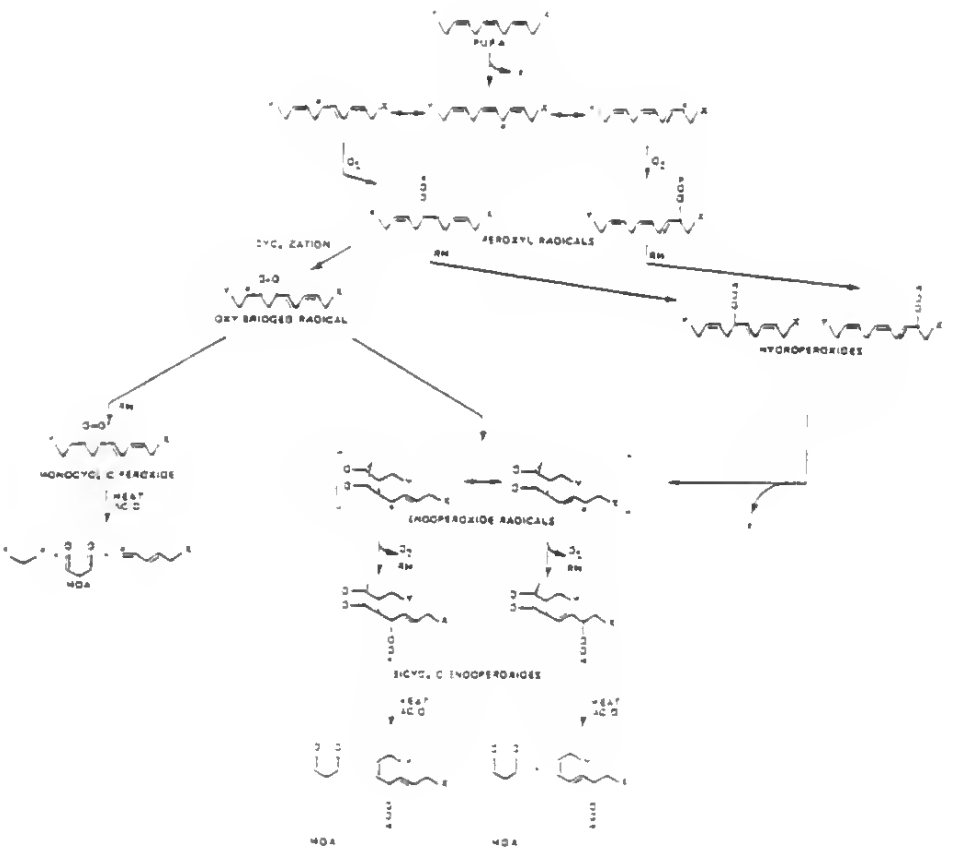


Figure 2. The chemical pathway summary of lipid peroxidation products maliondialdehyde and lipid hydroperoxides as described previously (47)

PUFA also leads to the formation of 4-hydroxynenal (4HNE), a toxic aldehyde among others, that potentiates cytotoxic oxidation processes (98).

Furthermore, lipid peroxidation is associated with decreased membrane integrity, and may in fact assist in "labeling" that affected cell as a target for neutrophilic attack (48, 58). Recent evidence also suggests that greater formation of hydroperoxides can initiate events that lead to apoptosis (34) Although it is beneficial to increase the fluidity of cellular membranes to an optimal physiological level to enable recycling of receptors or improve membrane-bound enzyme movement, fluidity that compromises cellular integrity is dangerous for all cellular functions.

Obesity and Oxidative Stress

Evidence from our laboratory suggests that obesity is associated with an increase in myocardial oxidative damage as evidenced by increased levels of by-products of lipid peroxidation (111). Damaged lipids can alter cellular homeostasis, deteriorate contractile function or cause cell death (105). Substantial indirect evidence suggests that there are several potential factors that may contribute to myocardial lipid peroxidation in the obese.

Increased Myocardial Work Rate

Obesity is characterized by an increased mechanical load on the heart due to increased fat, total body mass, and peripheral resistance (7). Hyperinsulinemia appears to

elevate blood pressure by activating the renin-angiotensin system or renal tubules directing to retain sodium, by stimulating arterial smooth muscle hypertrophy, or by altering the ionic (Ca^{2+}) efflux from the smooth muscle cells (88). Blood pressure is elevated, and stroke work is increased (2). Very recent evidence has shown that leptin, the adipocyte-derived hormone, can act (1) through the central nervous system to either increase the sympathetic nerve activity and vascular resistance in the kidney, (2) a decrease in renal plasma flow, and/or (3) an increase in heart rate (43, 70) Together, these factors appear to increase the rate pressure product and myocardial oxygen consumption (15). Theoretically, the oxygen flux through the mitochondrial respiratory chain would be increased in response to this workload. Animal studies indicate that blood pressures are elevated in obese Zucker rats or rabbits fed either Purina chow diets or high fat diets (14, 18, 54, 55, 64). Human studies also report elevated blood pressures and/or heart rates in obese humans (2). This increased work rate of the heart would increase myocardial oxygen consumption, and subsequently, oxygen flux through the mitochondrial electron transport chain.

Studies have reported elevated mitochondrial oxidative capacities in hearts of obese mice (12) and mitochondrial lipid oxidation providing indirect evidence for the notion that oxygen flux through the mitochondria may be a primary source of ROS production (76). Some investigators report that maximal state 3 respiration (i.e., ADP-stimulated) is greater in cardiac mitochondria from older obese (ob/ob) mice when

compared to lean mice (12). These data have been interpreted as an indication that obese animals have tighter mitochondrial coupling, and therefore a greater rate of O_2 consumption (76). As mentioned earlier, it is well established that increased O_2 uptake and electron flux through the electron transport chain leads to increased $O_2^{\cdot}$ formation (114).

Compromised Antioxidant Defense

Myocardial oxidative damage may be the result of an insufficient cellular antioxidant defense (48). Reductions in antioxidant enzymes activities (SOD, GPX and CAT) or reductions in the level of dietary antioxidants such as vitamin E, β-carotene and vitamin C have been associated with increased lipid peroxidation and cellular damage (23, 24, 114). Lowered glutathione levels also contribute to lipid peroxidation (98). It is possible that any of these mechanisms alone, or in combination, could contribute to the elevated lipid peroxidation in obese animals.

Our initial experiment indicated that heart homogenates from genetically obese animals were more susceptible to oxidative damage. Specifically, an iron-mediated oxidative challenge *in vitro* resulted in greater production of thiobarbituric acid reactive substances (TBARS) in heart homogenates of obese Zucker animals (111). These findings may suggest that the antioxidant defense may not have been sufficient to protect against the oxidative stress incurred by obesity.

Obesity lowered plasma levels of antioxidants such as vitamin E and β-carotene in obese children and adults (24, 75, 79). In addition, this lowered antioxidant status was associated with increased plasma levels of PUFA in obese children (23). The relationship between myocardial lipid content and antioxidant status in various obesity models is not well documented.

Tissue antioxidant enzyme activities are also altered in various obesity models. Our initial study indicated that there were no differences in the activities of the primary antioxidant enzymes within the heart, CAT, GPX and CuZn-SOD (111). However, the activity of the Mn-SOD isoform was elevated in hearts of these obese Zuckers. Other studies report reduced myocardial CAT and GPX activity (units/g heart tissue) in gold-thioglucose-induced obese mice (12). In contrast, investigators treating lean rats with obesity-inducing high fat diets using sources such as PUFA (corn or fish oils) or lard have shown increased liver GPX activity with no change in SOD (107), or unaltered hepatic antioxidant enzyme activities (45). Lean Wistar rats were fed a variety of n-3 fatty acid high fat diets, and myocardial total and Mn-SOD and GPX values were found to be lower than compared to rat chow fed controls; urinary and tissue TBARS values were also greater compared to those of chow-fed controls (62). Clearly, these findings regarding the effects of obesity on antioxidant enzyme activities are divergent. To provide clarity to the question whether myocardial antioxidant enzyme activities are sufficient to protect against

the stress of obesity, this study will measure SOD, GPX and CAT in both high-fat fed and fa/fa obese animals.

GSH levels appear to be altered in obesity. Glutathione is depleted (reduced by 45%) in the livers of overfed, overweight animals, and synthesis rates are reduced by 40% (93). These data are corroborated by findings which indicated that liver glutathione stores are depressed in overfed, obese mice (92). Furthermore, these animals were more susceptible to allyl alcohol-induced injury and necrosis. Interestingly, the obese Zucker rat resists drug-induced hepatotoxicity, and this has been correlated with this genotype's higher glutathione levels compared to their lean counterparts (9). Our initial study indicated that the myocardial non-protein thiol level (90% glutathione) was higher in obese Zucker rats compared to lean, though this value was not found to be significant. These contrasting findings do not elucidate the relationship between GSH and obesity-induced myocardial lipid peroxidation. Hence, this is justification to assess the thiol content (GSH) of the myocardial tissue of genetic and overfed obese animals to determine whether alterations in GSH content can contribute to the elevated lipid peroxidation in obesity.

In summary, there is no definitive evidence to support that the antioxidant defense is compromised in the myocardium of obese animals. This forms the rationale to compare the antioxidant enzyme activities and GSH content of myocardial tissue from two models of obesity in this experiment.

Myocardial Fat Composition and Oxidizability

Findings from other studies report that significant correlations exist between the type of tissue lipid and degree of lipid oxidizability *in vivo* or *in vitro* (109). Increased myocardial PUFA increases the risk for oxidative attack. Specifically, greater serum formation of TBARS occurs in the presence of triglycerides compared to high-density lipoprotein (HDL) cholesterol (85). Lipoprotein oxidizability is also influenced by lipoprotein composition, such that PUFA are more readily oxidized than monounsaturated fats (107, 113). Whether the enhanced oxidizability of the tissue lipids in obesity is due to increased oxidant challenge, decreased antioxidant defense, or altered phospholipid composition requires further investigation (109).

Fatty infiltration of the myocardium increases the risk for lipid oxidation by providing more fat substrate targets for oxidation (63). In addition, dietary consumption of PUFAs or saturated fats can affect the fatty acid composition of the phosphatidylcholine or ethanolamine molecules in total heart membrane phospholipids (62, 63). Considering the PUFA target for oxidative damage can be enhanced by dietary consumption of fats, this may in part explain the greater susceptibility to myocyte damage in obesity (57).

Both human and animal investigations have reported reduced antioxidant levels in tissue and plasma of obese subjects (19, 24, 91). Swine fed a diet enriched in fish oils or

other 18:3 oils showed mild symptoms of vitamin E deficiency; following supplementation with vitamin E and selenium, these symptoms disappeared (19, 91).

It is unknown whether the high-fat diet typically consumed by obese humans which consists of ~45% fats (approximately 18% saturated fats, 16% monounsaturated fats and 6% PUFAs) (27) renders the myocardium susceptible to lipid peroxidation *in vivo* or *in vitro*. It is also unclear how this type of diet affects antioxidant enzyme activities and GSH levels in myocardial tissue. This experiment will address this issue by feeding lean animals the typical fattening diet, and assessing lipid peroxidation and susceptibility to oxidative damage *in vitro*.

The Genetic Contribution to Obesity

The obesity phenotype cannot be simply reduced to Mendelian segregation patterns largely for two reasons. First, body fat content and excess body fat results from an intricate network of interactive causes that may be related to specific DNA sequencing but are also a function of behavior and lifestyle (11). In addition, obesity is also a heterogeneous phenotype, and there is growing evidence that a certain phenotype is modified by other causal factors. Considering that human obesity classification schemes consist of a minimum of four different phenotypes, each with different cases, it is difficult to identify the etiology.

Any contribution of a genetic effect on caloric intake in human appears to be minimal. Familial correlations computed in different relative types from the Quebec Family Study revealed that there were no significant heredity effects on caloric intake (10, 11). However, when intakes of carbohydrate, fat and protein were expressed in a percent of total energy intake, the contribution of genetic factors increased (11). These results suggest that macronutrient selection may be under genetic control, and could indicate susceptibility of some individuals to be in positive energy balance over a long period of time. It is also possible that differential genetic expression of specific (neuro) peptides that modulate or inhibit food intake are the underlying basis for individual variations in energy and macronutrient intakes (11). For example, over expression of stimulatory neuropeptides such as norepinephrine, neuropeptide Y, galanin and endogenous opiates increase food intake, and elevated levels of serotonin, histamine, neurotensin, cholescytokinin, glucagon, insulin and corticotropic releasing hormone suppress food intake (100).

Among the animal studies that employed high-fat or calorically dense diets to fatten animals and simulate diet induced hyperphagia, there is much heterogeneity in dietary treatments. The result is a wide range of physiological responses differential energy intakes based on fat or sugar content of the diet and animal model (14, 42, 55, 62, 95). Recently, investigators have designed specialty diets to identify animals that are "susceptible" to obesity (64). Outbred Sprague-Dawley rats show differential responses to

a condensed milk diet such that half of the rats became obese, and some gained moderate fat weight (64). Other studies show that genetically inbred Zucker rats possessing the heterozygous Fa/? genotype also respond to fattening diets within 3-4 weeks (33). The responsive nature of these susceptible animals has been attributed to a number of factors including alterations in expression of satiety factors (leptin) and lipogenic enzymes, improved energy efficiency (improved ability to utilize less energy per unit food intake, fewer energetic futile cycles), and increased sensitivity to insulin that would promote more rapid fat deposition in response to calorically dense diets (11).

It is unknown whether the leptin receptor defect in the fa/fa obese Zucker rat increases the risk for myocardial oxidative stress. Intuitively, oxidative stress would be elevated in any myocyte with elevated rates of oxidative phosphorylation (i.e., myocytes that function against a greater workload) regardless of the single genetic defect. However, there may be a greater risk for oxidative stress in human or animal subjects with high heritability for obesity. It is well established that familial predisposition, or genetics, is a primary risk factor for cardiovascular disease, and that heart disease is complicated by obesity (7). Considering the important and significant role of the genetic influence upon the development of obesity, it is also essential to examine the influence of the fa/fa genotype in our investigation.

Preliminary Experiments

Our laboratory has recently performed initial experiments investigating the relationship between myocardial oxidative stress and obesity (111). Using the fatty Zucker rat animal model, hearts from 12 month old lean (590 ± 60 g, Fa/?) and obese (881 ± 56 g, fa/fa) males were analyzed for a series of antioxidant and lipid peroxidative characteristics. The data revealed that the hearts from the obese animals had higher basal levels of lipid hydroperoxides and thiobarbituric reactive acid substances. We performed experiments to compare the primary enzymatic, non-enzymatic and tertiary antioxidant defense within the heart.

Susceptibility to an Oxidative Challenge *In Vitro*

Production of lipid peroxidation was exacerbated in the homgenized heart tissue of obese animals compared to the lean following an iron-induced oxidative challenge *in vitro*. These data indicate that the myocardial tissue from obese animals had higher basal lipid peroxidation, but also a higher level of hydroperoxides per unit lipid following the same exposure to oxidative reagents (ferric chloride) in an *in vitro* bath. We attributed this susceptibility to oxidative damage to either: (1) a compromised antioxidant defense within the myocardium, or (2) an increased lipid substrate availability within the myocardium of obese animals.

Primary Antioxidant Defense

The left ventricular myocardial samples from the obese animals did not have higher antioxidant enzyme activity levels compared the lean animals. Specifically, catalase (CAT), glutathione peroxidase (GPX), total superoxide dismutase (SOD) and the copper-zinc dependent SOD activities were not higher than those in the myocardium of the lean animals However, the manganese-dependent isoform of SOD (Mn-SOD) in the obese animals showed significantly higher activity compared to the Mn-SOD activity in the lean rats. The myocardial non-protein thiol content (representative of glutathione) was not significantly different between the groups (14.1 ± 1.1 lean, 16.2 ± 2.0 obese).

Tertiary Antioxidant Defense

Evidence exists to support the notion that heat shock proteins (HSP)s of the 72 kD family provide protection against oxidative stress *in vitro* and *in vivo* (20, 66). In our preliminary study, we measured the relative contents of the constituitive isoform of HSP 73 kD, and the inducible HSP 72 kD. Our data revealed that the relative contents of both of these HSPs were not significantly different between the lean and the obese groups.

Lipid Content of the Myocardium

The lipid content of the hearts from obese animals was nearly double that of the lean animals (59% versus 33% of the total wet mass). Others have suggested that elevated

lipid peroxidation may be the result of increased lipid substrate availability (34, 63), and our data appeared to be in agreement with this postulate

Unanswered Issues

Our data suggest that elevated lipid substrate and an insufficiently up-regulated antioxidant defense are important issues in obesity induced myocardial oxidative damage. However, there are other interpretations of these data that require investigation. First, the elevated Mn-SOD activity suggests that either oxidative respiration is occurring at a higher rate in the obese animals compared to the lean (i.e., greater heart work), or there is an increased rate of superoxide production at the site of the mitochondrion to cause up-regulation of the enzyme. Second, the antioxidant defense also includes dietary antioxidants which are important in preventing lipid peroxidation, inlcuding vitamin E and β-carotene. These dietary antioxidant characteristics of the myocardial tissue of these fatty Zucker animals have not previously been measured It is possible that obesity elevates the level of oxidative stress such that the tissue vitamin E and β-carotene levels may not be sufficient to scavenge the ROS generated in the myocardial tissue. Third, it is possible that this elevated lipid peroxidation may be in part due to the leptin receptor defect of the Zucker strain. All the perturbations that are associated with the genetic defect of the Zucker strain have not yet been elucidated, and it is important to address whether the fa/fa

genotype or diet-induced obesity in heterozygotes (Fa/?) is more important in contributing to myocardial oxidative stress.

CHAPTER 3
METHODS

Animals

Male lean and obese fatty Zucker rats (7 weeks of age) were used in this experiment. This age of rat was chosen due to the inability to separate animals by body weight between the lean and obese (fa/fa) groups (13, 18). Males were used to prevent any possible antioxidant-protective effect of estrogen in females (114). Sample sizes are based on a statistical power analyses performed using data from our preliminary experiment (Appendix A). Animals were individually housed, maintained on a 12:12 hour light:dark cycle. Following a one-week adjustment period, the experimental diets were administered.

Experimental Design and Diet

The control diet contained the National Research Council's recommended daily nutrient intake for rats (77, 84). High sugar or high fat diets have been widely used to fatten animals within 3-4 weeks, with results being more pronounced at 8-10 weeks (33), and have been characterized as highly palatable to rats (68, 89, 95). This study will employ a high-fat, high-refined sugar diet in an effort to most closely mimic the diet consumed by obese Americans (27, 83). This type of diet was also chosen in the effort to induce

voluntary hyperphagia. Data from theFramingham and Lipid Research Clinics (LRC) projects (27, 83) and other cohort studies (6, 35, 110) indicate that the typical diet

Table 1. Schematic of Experimental Design using the fatty Zucker Rat Model

LEAN (Fa/?)	Control Diet	n=15
LEAN (Fa/?)	High-Fat Diet	n=15
OBESE (fa/fa)	Control Diet	n=15

consumed by obese humans is comprised of ~15% protein, ~ 39-50% total fats (~18% saturated fat of animal origin, 6% polyunsaturated fats, 16% monounsaturated fats with unsaturated/saturated ratios ranging from 0.24-0.4) and ~37-42% carbohydrates (15-20% from added or simple sugars). To determine whether obesity elicits an alteration in the antioxidant status of obese animals, dietary antioxidant concentrations were the same per kg food, and the daily antioxidant intakes with feeding were retrospectively calculated. Therefore, in this study, lean animals (Fa/?) were fed a calorically dense diet for a period of nine weeks, as this time period appears to be adequate to induce significant obesity in high-fat fed groups (33, 107). The diets for all three groups were prepared by Research Diets Inc. (New Brunswick, NJ). The macronutrient percentages of the diets are contained in Table 2. Specific macronutrient and micronutrient composition of the diets are in Table 3. Animals were allowed to eat food and water ad libitum, and caloric intake was monitored daily (food volume in g, and total calories consumed.

Animal Model Justification

This study used an animal model comparable to human obesity, the fatty Zucker rat. This animal was chosen because: (1) the invasive nature of these experiments prevents the use of human subjects; (2) the fatty Zucker rat is a genetic model of obesity possessing similar symptomology as humans including hypertrophy and hypertension (13); (3) its widespread acceptance as a model for investigating human obesity; and (4) fatty Zucker animals demonstrate depressed ventricular function similar to that observed in human cases of obesity, such that responsiveness to β-adrenergic stimulation is reduced and the pressure developing ability of the ventricle is reduced (lower peak systolic stress at any given volume)(15, 82).

The overfed, overweight rat model has been cited as the best animal model to use in any pharmacokinetic research (16) and studies of human hypertension. Therefore, both the genetic (fa/fa lepitn receptor defect) and overfed (high-fat), overweight animal model will be used in this investigation

Assessment of Systemic Changes With Obesity

Several important physiological variables were measured before, during, and following the dietary treatment period. These measures included resting body weight,

Table 2. Description of the control and high-fat, high-carbohydrate diet constituents fed to lean and obese Zucker rats.

Control Diet (D12450)		Fattening Diet (D12451)	
Protein	15%	Protein	15%
Carbohydrate*	75%	Carbohydrate**	45.1%
Fat	10%	Fat	44.9%
Total	100%	Total	100%

Largely complex sugars (2/3 cornstarch) **Added ~10% refined sugars (2/3 sucrose)

Table 3. Macronutrient and micronutrient composition of the diets fed to the experimental groups of lean and obese fatty Zucker rats.

Ingredient	g/kg diet	Ingredient	g/kg diet
Casein, 80 mesh	150.00	Casein	150.00
L-Cysteine	3.00	L-Cysteine	3.00
Cornstarch	250.00	Cornstarch	72.80
Sucrose	350.00	Sucrose	172.80
Soybean Oil	25.00	Soybean Oil	25.00
Lard	20.00	Lard	177.50
S10026	10.00	S10026	10.00
L-Cysteine	3.00	L-Cysteine	3 00
Dicalcium Phosphate	13.00	Dicalcium phosphate	13.00
Calcium Carbonate	5.50	Calcium carbonate	5.50
Potassium Citrate, 1 H_2O	16.50	Potassium Citrate, 1 H_2O	16.50
Vitamin Mix (V10001)	10.00*	Vitamin Mix (V10001)	10.00*
Choline bitartate	2.00	Choline bitartate	2.00

*See Appendix B for details of Vitamin Mix constituents.
(Soybean oil is 14% saturated, 23% monounsaturated, 51% linoeic acid and 7% linolenic fatty acid.)

oxygen consumption, heart rate, blood pressure, and blood glucose and insulin levels. Experimental details for each measure follow

Resting Oxygen Consumption (VO_2)

Body masses were recorded at the beginning of the study and weekly thereafter until sacrifice. Resting oxygen consumption (VO_2) of each animal was assessed at the conclusion of the feeding treatment to determine differences between groups (32). Oxygen consumption was measured by open-circuit spirometry using a specially constructed, sealed metabolic chamber (5 X 6 X 5 cm, Truemax gas analyzing system). Animals remained in the chamber with oxygen consumption measured upon equilibration of the gas in the chamber (~40 min). Flow rates for gas sampling were set at 0.3 L/min., and resting VO_2 was estimated using the following formula: (flow rate)(% O_2 difference between the ambient air and the chamber)/ body mass (kg) = VO_2 in ml/kg/min.

Heart Rate and Blood Pressure

Systolic blood pressure (BP) and heart rate (HR) in awake, conscious animals were assessed in all animals. A tail pressure cuff system (Kent Scientific, model #s BP1001, BP1004) was used to determine systolic blood pressure. HR was determined using this same apparatus by allowing the piezoelectric transducer to detect the pulsations of blood flow within the proximal region of the lateral tail vein. The analogue signal was directed through a pre-amplifier and A/D converter to a pen chart recorder (Grass

instruments). One lean and one obese animal were tested simultaneously to reduce any experimental variations between testing sessions. Prior to any data collection, animals were placed into the warming restrainer on three different occasions for a period of 30 min to acclimate them to the procedure and reduce any inflation of the true BP. BP and HR measures were collected prior to the feeding treatment, once each week, and at the conclusion of the feeding period.

Blood Glucose and Insulin Concentrations

Immediately prior to sacrifice, a ~5mL blood sample was obtained from cardiac puncture. Fresh blood samples were collected using EDTA treated vacutainer tubes. Plasma was separated by centrifugation and immediately frozen for later analysis of blood glucose and insulin levels. Blood glucose was assessed using an enzymatic, colorimetric technique (101). Insulin levels were assessed by a radioimmunoassay technique described previously (5)(commercial kit, LINCO Research ^{125}I label). Glucose samples were performed in duplicate, and insulin samples were performed in quadruplate. The average of two hematocrits values were recorded as the sample score.

Heart Weight

Immediately following sacrifice, the hearts were rapidly excised and placed in aerated ice-cold modified Kreb's solution to remove the remaining blood. Following excision of the papillary muscles, the hearts were blotted and weighed immediately.

Adiposity

A BMI equivalent for rats, the adiposity index, was performed in all animals using a well-documented method (65). Briefly, the length of the rat from the tip of the nose to the anus was measured. The body mass and the length of the animal was calculated using the formula: Adiposity Index = the cube root of the body mass (g)/ length (mm) X 10^4. The adiposity index was determined immediately prior to sacrifice.

Heart Tissue Composition

To determine whether the dietary treatment affected heart tissue composition, the fat, water composition and dry weight of the hearts of lean and obese animals were determined as follows.

Lipid Content of the Myocardium

A modified version of an earlier extraction technique was used to isolate myocardial lipid (30). Briefly, heart samples were homogenized in a methanol: chloroform

mix (2:1 v/v) for 2 min at room temp. Samples were centrifuged for 4 min at 400 X g. Supernatants were decanted, and the pellet was resuspended and re-extracted with methanol:chloroform:0.2 N HCl (2:1:0.8 v/v). The two phases were separated by another 2 min centrifugation at 400 X g. The supernatants were pooled and the phases were separated by a third 4 min centrifugation at 400 X g. The lower chloroform phase was removed and neutralized by drop-wise addition of methanolic NH_4OH. Samples were concentrated under a stream of nitrogen. The weight of the residue was recorded as the amount of lipid mass per unit heart weight.

Water Content and Dry Weight

To determine the myocardial water content and dry weight, a piece of ventricular tissue was cut and placed in a pre-massed tube. The sample's weight was recorded. The sample was freeze-dried in an evaporator at a negative pressure of 10^{-1} mmHg, and re-weighed to obtain the sample's dry weight (tissue protein/lipid). The water weight of the sample (tissue water) was calculated by: wet weight- post-drying weight.

Radical Production by the Myocardium

To determine whether obesity affects the respiration rates or the radical production (specifically $O_2^{\cdot-}$) of myocardial tissue papillary muscles were isolated and stimulated *in vitro*, and an indirect assessment of ROS production was performed.

Isolated Papillary Muscle Experiments

To determine whether hearts from obese animals generate ROS at greater rates, papillary muscles were isolated from hearts from all animals as described previously (15). In brief, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). After reaching a plane of surgical anesthesia, the chest of the animal was opened, exposing the contracting heart. The heart was subjected to cardioplegic arrest by infusion of ice cold modified Kreb's Hensleit buffer (all in mmol/L: NaCl 115.0, $NaHCO_3$ 20.0, KCl 4 0, K_2HPO_4 0.9, $MgSO_4$ 1.1, CaCl 2.5, and glucose 11.0) by a syringe through a cut in the aortic root. Ice cold buffer was applied to the heart topically to assist in cooling of the organ. After flushing the heart with buffer, the heart was removed from the animal and placed into an iced tissue bath with modified Kreb's buffer bubbled with 95% O_2/5% CO_2. Under a magnifying glass, a papillary muscle was rapidly excised and tied with sutures on both ends. The muscle was transferred to a small tissue chamber containing warm Kreb's buffer (37°C, 30 ml) bubbled with the same gas mixture. One suture end was fixed to the chamber, and the other end fixed to a force transducer (Grass Instruments, Model #FT10). A specially designed pair of platinum electrodes provided field stimulation to the muscle at the following parameters: 100V, 50Hz, 2 ms duration and 3 pulse per sec (to simulate 180 bpm). Following a 15 min equilibration period, the muscle was stimulated for a 30 minute time period. To determine

whether $O_2^{\cdot-}$ anions are formed at greater rates in response to electrical stimulation, the bathing medium also contained 10^{-5} M cytochrome C (C-2506, Sigma Chemical). Cytochrome C is reduced as a function of superoxide production. The tissue bath was wrapped in foil and the experiments were conducted in a dimmed laboratory to reduce photoreduction in ambient light. Following the 15 min stimulation treatment, the bathing medium was collected and analyzed spectrophotometrically for the reduction of cytochrome C as previously described (87). The magnitude of absorbance change at 550 nm reflected the amount of $O_2^{\cdot-}$ in the bathing medium (82). To confirm that the assay was detecting $O_2^{\cdot-}$ production, hypoxanthine (5×10^{-4} M; H-9377 Sigma Chemical) was added to xanthine oxidase (0.02 U/ml; X-4500 Sigma Chemical) to produce $O_2^{\cdot-}$ that reduced cytochrome C. Alternatively, native SOD (10^3 U/ml; S-7008 Sigma Chemical) was added to the medium to test inhibition of the radical.

Assessment of Myocardial Antioxidant Status

To determine whether obesity or the overfeeding treatment affected oxidative and antioxidant capacity, left ventricular samples from all groups were assessed for oxidative and antioxidant enzyme activities.

Oxidative and Antioxidant Enzyme Activity

Citrate synthase (CS; EC 4.1.3.7) activity was used as a marker for oxidative capacity using a method previously described (99). Superoxide dismutase (SOD; EC 1.15.1.1), selenium glutathione peroxidase (GPX; EC 1.11.1.9) and catalase (CAT; EC 1.11.1.6) activities were used as markers for antioxidant capacity using previously described procedures (1, 29, 81). All assays were performed in duplicate and on the same day to reduce interassay variation. Activities were normalized to protein in the sample using previously described spectrophotometric dye binding methods (36, 111).

Tissue Thiol Measurements

Tissue thiols are molecules that contain sulfhydryl groups. They are important in the regulation of both cellular redox status and antioxidant capacity (37). Therefore, total, protein and non-protein thiols from the left ventricle were assayed from all experimental animals. Thiol content was determined spectrophotometrically using a previously described DTNB-based technique (50). Since glutathione is the dominant non-protein thiol in the cell, this measure was used as a marker of tissue glutathione levels (49).

Biochemical Indicators of Oxidative Stress

To determine the amount of radical-mediated oxidative damage in the heart, left ventricular levels of two by-products of lipid peroxidation were measured.

Lipid Peroxidation Measurements

Malondialdehyde levels were determined spectrophotometrically using the thiobarbituric acid-reactive substances (TBARS) method previously described (108). The agent 1,1,3,3-tetraethoxypropane was used as the standard for this assay. Samples were performed in duplicate.

Lipid hydroperoxides were quantified using the ferrous oxidation/xylenol orange technique previously reported (44). Cumene hydroperoxide was used as the standard for this assay. In our laboratory, the coefficients of variation for the TBARS and lipid hydroperoxide assays are ~3 and 4 percent, respectively All samples were performed in triplicate.

Oxidative Challenges *in vitro*

To investigate the relationship between obesity and myocardial antioxidant capacity, heart homogenates from animals in all groups were subjected to a series of several different ROS-generating systems A section of the left ventricle from each heart was homogenized in 0.9% saline at a concentration of 10:1 in nitrogen gassed 50 mM potassium phosphate buffer at pH 7.4 according to a previous method (40). Aliquots of the homogenates were incubated at a concentration of 10 mg protein/ml in the presence of

an ROS generating system. Following each challenge, the homogenates were analyzed for lipid peroxidation using the previously mentioned technique (108).

Xanthine-Xanthine Oxidase System (Superoxide Generator)

Superoxide radicals were generated by the reactions involved in a xanthine-xanthine oxidase system similar to an earlier method (87) One ml of 1 mM xanthine and 0.1 IU xanthine oxidase were added to a 1 ml aliquot of heart homogenate and incubated at 37°C for 15 min

Hydrogen Peroxide System

One ml of hydrogen peroxide (100 mM) was added directly to a one ml aliquot of heart homogenate and incubated at 37°C for 15 min according to a previous method (96).

Ferric Chloride System (Hydroxyl Generator)

Hydroxyl radicals were produced in the heart homogenates by adding 0.1 mM ferric chloride ($FeCl_3$) and 1 mM ADP. The choice of these particular concentrations is based on previous (3) who found that this concentration of iron-ADP induced free-radical mediated arrhythmias in the isolated perfused rat heart and that it was possible to prevent these arrhythmias by perfusing the heart with SOD (3).

AAPH System (Peroxyl Generator in the Lipid Phase)

Peroxyl radicals were generated in the aqueous phase of homogenate by thermal decomposition of 2,2'azobis(2-amidinopropane)-dihydrochloride, (AAPH). 1ml of AAPH solution and 1ml of heart homogenate will be mixed in and incubated at 37°C for 2 hrs.

Following incubation of the heart homogenates in each system, 200 mM butylated hydroxytoluene (BHT) was added to stop the oxidative reaction TBARS formation and lipid hydroperoxide concentration were then analyzed as previously described (108).

Statistical Analysis

All dependent measures (antioxidant and biochemical parameters) were subjected to a one-way analysis of variance (ANOVA). Significance was established at $p<0.05$. In the case of significant differences, Scheffé post-hoc analysis was performed to determine where differences existed. Bivariate correlations were performed between TBARS and hydroperoxide levels and systemic, dietary and biochemical measures to determine any relationships between lipid peroxidation and these variables. Furthermore, a stepwise (forward) regression was performed on select variables to determine which variables contribute most to lipid peroxidation in both models of obesity.

CHAPTER 4
RESULTS

Due to the variance between the adiposity levels attained within experimental groups, each of the three groups of animals were separated into two groups based on adiposity: low BMI and high BMI. BMIs that were above the group average were defined as "high" and BMIs that were below the group average were defined as "low". Therefore, the following annotation will be used throughout the remainder of the manuscript:

CONTROLS:	Low BMI = C-L-BMI
	High BMI = C-H-BMI
HIGH-FAT FED:	Low BMI = F-L-BMI
	High BMI = F-H-BMI
OBESE:	Low BMI = O-L-BMI
	High BMI = O-H-BMI

Diet and Antioxidant Consumption

The weekly food intake of all groups during the nine weeks of feeding is shown in Figure 3. The total caloric intake and dietary consumption of vitamins A and E are contained in Table 4. Although the fat-fed groups F-L-BMI and F-H-BMI consumed less total food ($p<0.05$), the caloric intake of these groups was higher compared to C-L-BMI and C-H-BMI ($p>0.05$). This is due to the fact that the food density of the high-fat diet

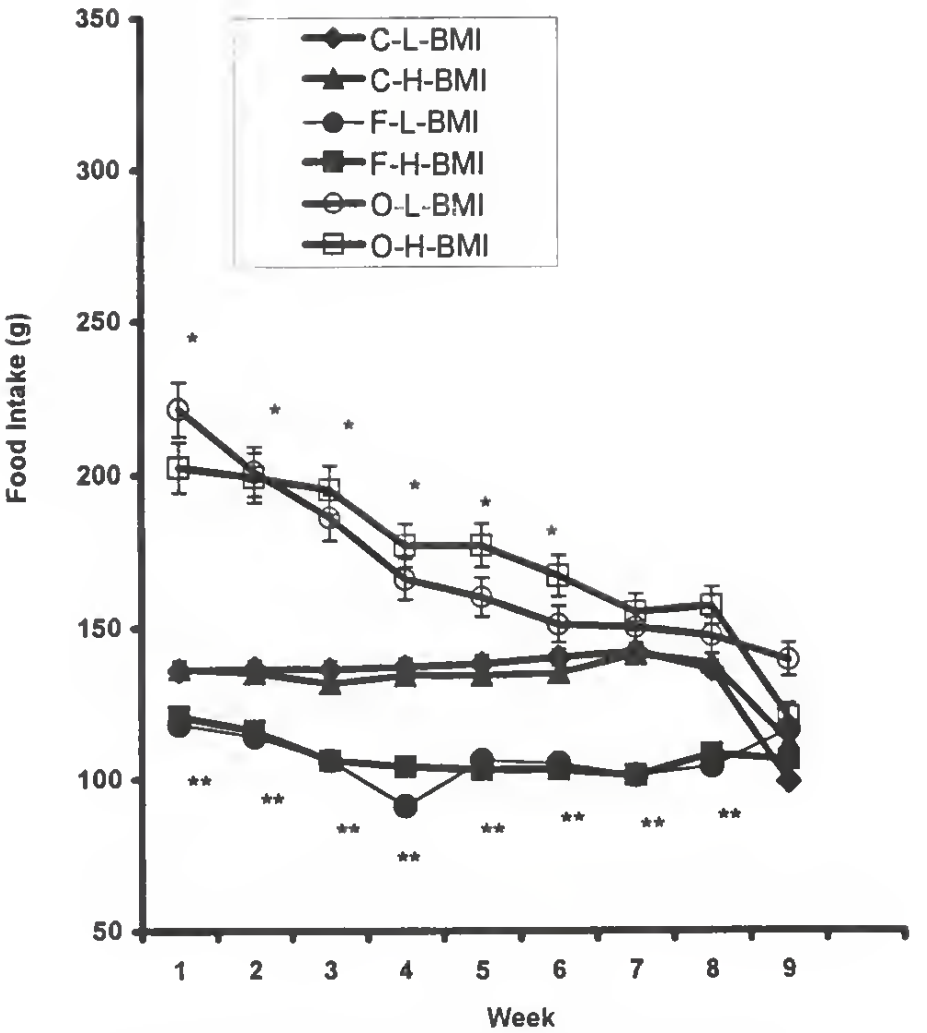


Figure 3. Food intake of the six experimental groups. Values are means ± SE. *Denotes greater than control $p<0.05$, and ** denotes less than controls at $p<0.05$.

was 4.74 kcal/gm compared to the diet consumed by the two lean groups (3.84 kcal/gm). The O-L-BMI and O-H-BMI animals consumed more total food and more calories than all other control and fat-fed groups ($p<0.05$). Vitamin E and A intake were a direct function of total food consumed (500 IU/ gm food vitamin E, 500,000 IU/ gm vitamin A). Therefore, groups F-L-BMI and F-H-BMI consumed less of vitamin E and A compared to the other four groups. In contrast, the obese groups O-L-BMI and O-H-BMI consumed the most of these two vitamins of all six groups ($p<0.05$).

Table 4. Total diet consumption, caloric intake and antioxidant intake of lean control, high-fat fed and obese Zucker rats during a 9-week feeding period. Values are means ± SEM. ** $p<0.05$ greater than groups 1-4, † $p<0.05$ less than all control and obese groups

Group	Diet Intake (g)	Calories (kcal)	Vitamin E (IU) (IU, x 10^5)	Vitamin A (IU) (IU, x 10^8)
C-L-BMI	1204 ± 34	4922 ± 85	6.02 ± 0.17	6.02 ± 0.17
C-H-BMI	1199 ± 29	5013 ± 83	6.0 ± 0.14	6.0 ± 0.14
F-L-BMI	1043 ± 18†	5661 ± 135*	5.21 ± 0.09†	5.21 ± 0.09†
F-H-BMI	1062 ± 18†	5682 ± 163*	5.31 ± 0.08†	5.31 ± 0.08†
O-L-BMI	1525 ± 43**	7199 ± 201**	7.62 ± 0.21**	7.62 ± 0.21**
O-H-BMI	1553 ± 56**	7330 ± 263**	7.76 ± 0.28**	7.76 ± 0.28**

where Vitamin E = vitamin E acetate, Vitamin A = Vitamin A Palmitate

Body Weight Changes With Feeding

Nine weeks of feeding the lean and obese Zucker rats resulted in a distinct separation between the body weights of the groups. Figure 4 contains the body weights data before, during and after the feeding treatment. The O-L-BMI and O-H-BMI were heavier than the other four groups at the start of the study, and gained weight rapidly during the first 4 weeks and were significantly heavier ($p<0.05$) than the other four groups at all time points during the feeding period. By week 6, the F-H-BMI became significantly ($p<0.05$) heavier than the control lean groups C-L-BMI and C-H-BMI and the fat-fed group, F-L-BMI. These body weight separations remained present throughout the remainder of the feeding period.

Morphological Characteristics

The morphological characteristics of the six groups are summarized in Table 5a. The O-L-BMI and O-H-BMI groups were characterized by a significantly ($p<0.05$) larger liver weights compared to all other groups. The heart weights were not significantly different between groups ($p>0.05$), but the heart weight/body weight ratio was significantly lower ($p<0.05$) in the groups O-L-BMI and O-H-BMI compared to all other groups. The diaphragm weight of the F-H-BMI was significantly greater than the weights of all other groups ($p<0.05$). The locomotor muscle weights are shown in Table 5b. The data indicate that the locomotor muscle weights of the soleus, gastrocnemius,

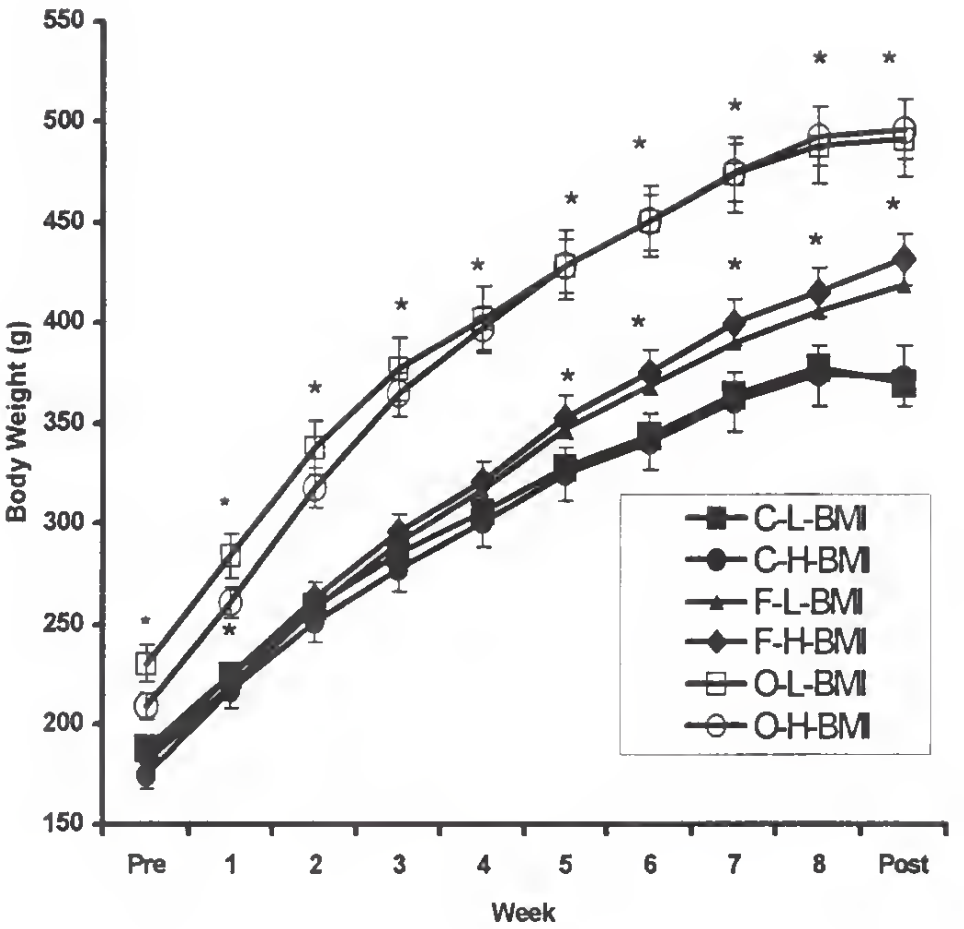


Figure 4. Body weight changes before, during and following the nine week feeding period. Values are means ± SE. * denotes greater than control at $p<0.05$.

Table 5a. Comparison of morphological characteristics between the six groups of Zucker rats following 9 weeks of feeding. Values are means ± SE., * p<-0.05 compared to all other groups, **p<0.05 greater than all lean control and fat-fed groups, † p<0.05 less than all lean control and fat-fed groups

	Heart Weight (g)	Heart/ Body Weight Ratio (g/kg)	Liver Weight (g)	Diaphragm Weight (g)
C-L-BMI	1.06 ± 0.03	2.91 ± 0.12	8.92 ± 0.40	0.86 + 0.02
C-H-BMI	1.05 ± 0.08	2.84 ± 0.16	9.99 ± 1.23	0.96 + 0.21
F-L-BMI	1.08 ± 0.08	2.59 ± 0.05	10.88 ± 0.85	0.93 + 0.07
F-H-BMI	1.17 ± 0.08	2.72 ± 0.06	10.54 ± 0.44	1.04 + 0.11*
O-L-BMI	1.10 ± 0.08	2.21 ± 0.06†	21.32 ± 1.15**	0.76 + 0.09
O-H-BMI	1.12 ± 0.05	2.53 ± 0.05†	22.11 ± 1.51**	0.79 + 0.05

Table 5b. Comparison of locomotor muscle weights between the six groups of Zucker rats following 9 weeks of feeding. Values are means ± SE. * p<0.05 greater than all other groups, † p<0.05 smaller than all other groups. All values are expressed in g.

	Soleus	Gastrocnemius	Plantaris	Tibialis Anterior
C-L-BMI	0.169 ± 0.006	1.42 ± 0.19	0.33 ± 0.02	0.711 ± 0.02
C-H-BMI	0.151 ± 0.014	1.39 ± 0.14	0.31 ± 0.03	0.647 ± 0.06
F-L-BMI	0.179 ± 0.008	1.45 ± 0.05	0.36 ± 0.01*	0.726 ± 0.02
F-H-BMI	0.184 ± 0.006*	1.63 ± 0.21*	0.37 ± 0.01*	0.755 ± 0.02*
O-L-BMI	0.112 ± 0.003†	1.03 ± 0.03†	0.21 ± 0.01†	0.415 ± 0.01†
O-H-BMI	0.119 ± 0.003†	1.05 ± 0.03†	0.22 ± 0.01†	0.432 ± 0.01†

plantaris and the tibialis angterior muscles of the obese groups O-L-BMI and O-H-BMI were significantly smaller ($p<0.05$) than those of their lean and high-fat fed counterparts in all other groups. The muscle weights of group F-H-BMI were significantly greater ($p<0.05$) compared to all other groups.

Physiological Characteristics

The physiological characteristics of the animals were recorded before, during and following the feeding protocol. These measures included resting heart rates and systolic blood pressures (double product of HR x BP = heart work), oxygen consumption, BMI and blood glucose and insulin concentrations.

Heart Rates, Blood Pressures, and Heart Work

The resting heart rates of the six groups did not differ ($p>0.05$) at any time point during the study (Figure 5a). However, the systolic blood pressures of the obese animals of groups O-L-BMI and O-H-BMI were significantly higher than those of all other groups during weeks 1-2 (Figure 5b). By week three, the fat-fed groups F-L-BMI and F-H-BMI exhibited a significantly higher ($p<0.05$) systolic blood pressure compared to groups C-L-BMI and C-H-BMI. This difference between groups persisted throughout the remaining weeks of the feeding period.

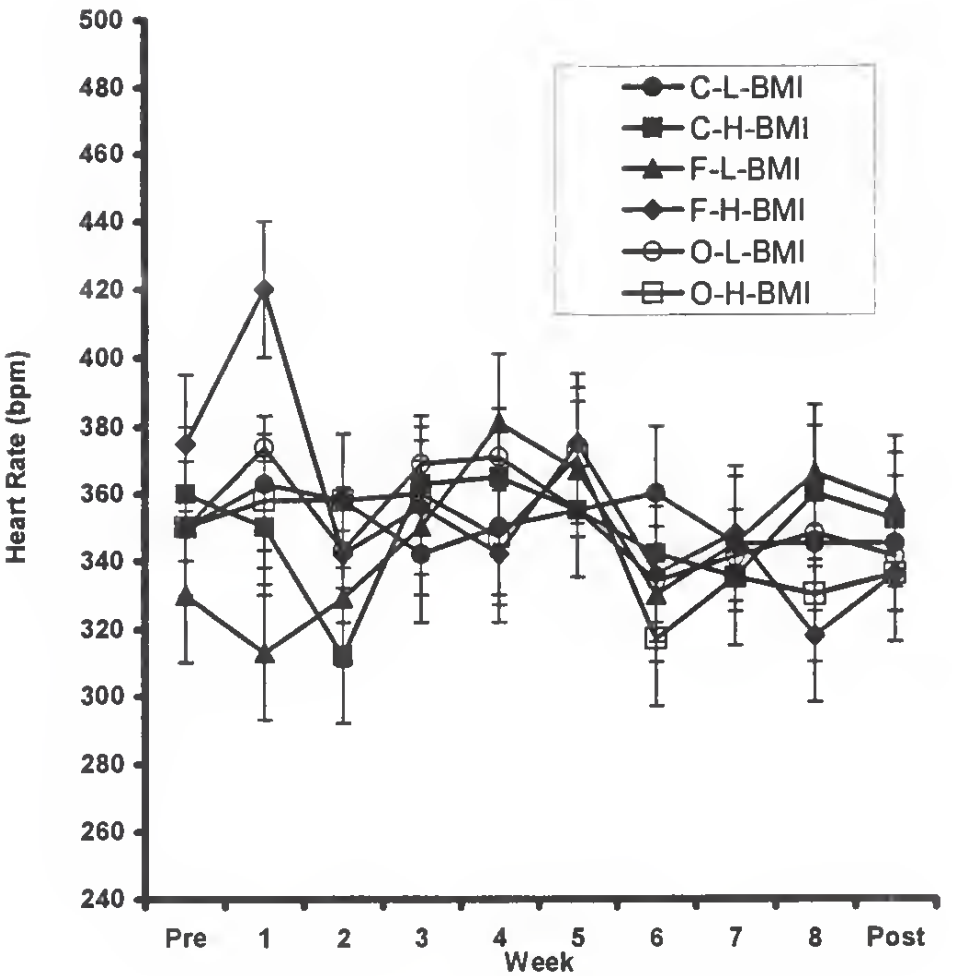


Figure 5. Cardiovascular functional measures.
5a) Resting heart rates of all experimental groups. Values are means ± SE.

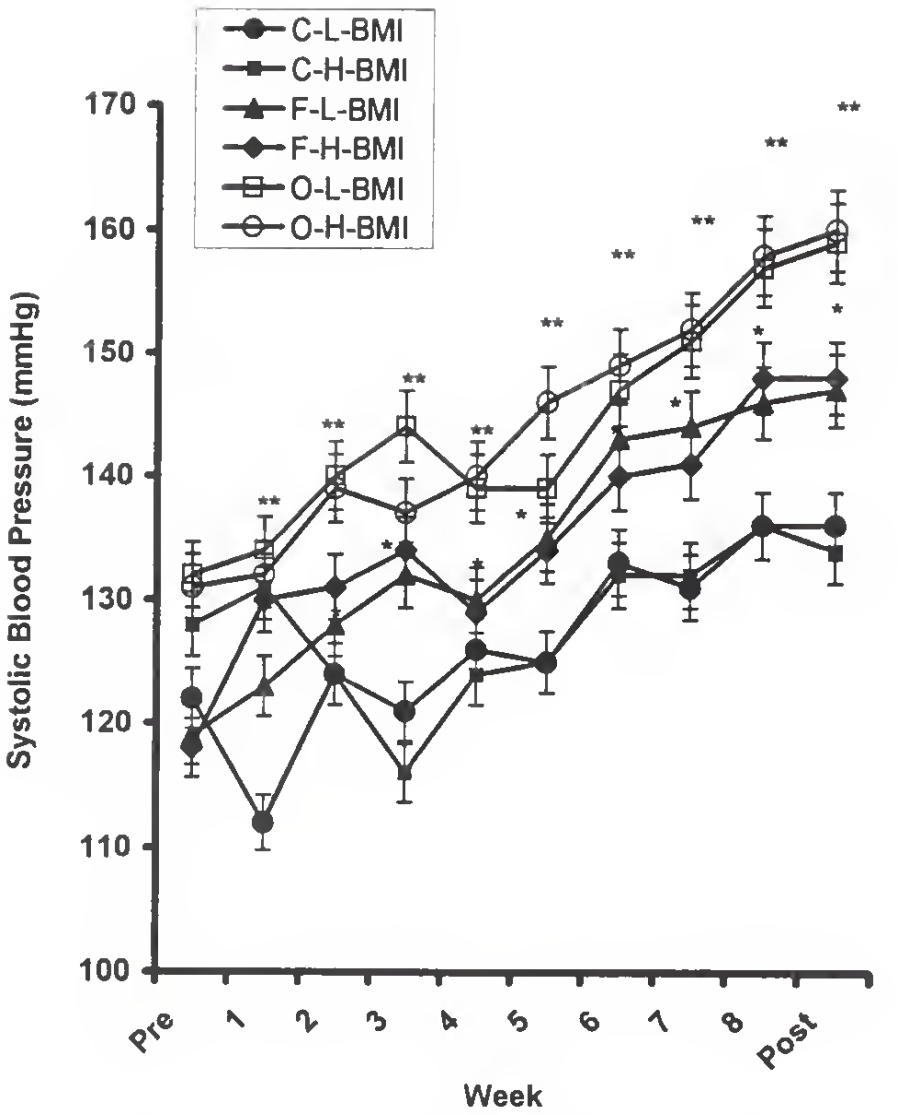


Figure 5. Cardiovascular functional measures.
5b) Systolic blood pressure in all experimental groups, Values are means ± SE. ** denotes different from fat-fed and controls, *denotes different from controls.

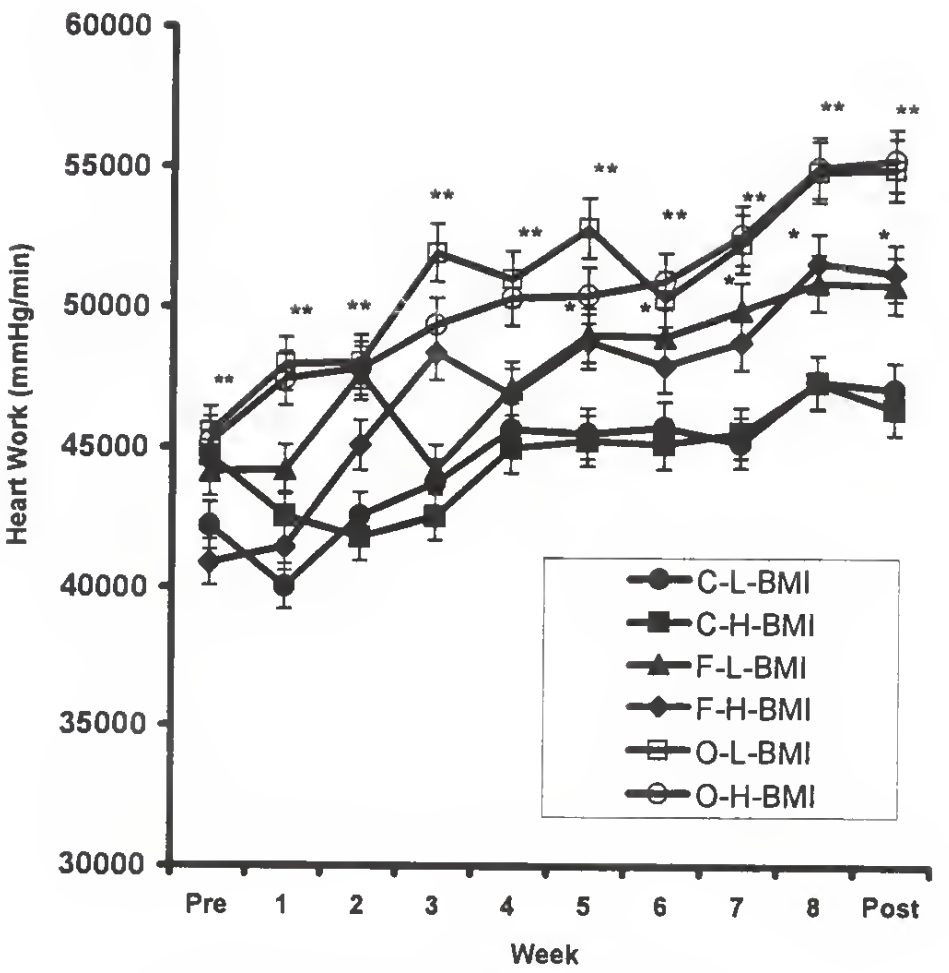


Figure 5 Cardiovascular functional measures.
5c) Myocardial work among the six groups. Values are means ± SE. * Denotes greater than control groups at $p<0.05$, ** greater than fat-fed and controls at $p<0.05$.

The rate pressure products of the heart rates X systolic blood pressures (defined as heart work) for each group during weekly assessments are shown in Figure 5c. The data indicate that the heart work generated by the obese groups O-L-BMI and O-H-BMI was significantly greater ($p<0.05$) than that of all other groups during weeks 1-7. By week 5, the heart work generated by the fat-fed groups F-L-BMI and F-H-BMI was significantly greater than that of lean control groups C-L-BMI and C-H-BMI. This difference between the six groups existed throughout the remainder of the study.

Oxygen Consumption and Body Mass Index (BMI)

Resting oxygen consumption (VO_2) and BMI values are shown in Table 6. The C-H-BMI group demonstrated a significantly higher ($p<0.05$) resting oxygen consumption value compared to all other groups, whereas, groups O-L-BMI and O-H-BMI showed the lowest Vo_2 values compared to the four remaining groups ($p<0.05$).

The BMI values following nine weeks of feeding resulted in significant differences ($p<0.05$) between the groups. The BMI values for O-H-BMI was greater ($p<0.05$) than all other groups, whereas the BMI values for groups F-H-BMI and O-L-BMI were greater ($p<0.05$) than groups C-L-BMI, C-H-BMI and F-L-BMI. C-L-BMI had the lowest ($p<0.05$) BMI value of all six experimental groups.

Table 6. Resting oxygen consumption and body mass index values (BMI) for all experimental groups of Zucker rats following 9 weeks of dietary treatment. Values are means ± SE. *p<0.05 lower than groups C-L-BMI, C-H-BMI, and F-L-BMI **p<0.05 greater than groups C-L-BMI and C-H-BMI, ***p<0.05 compared to all 5 other groups.

Group	VO_2 (ml*kg*min)	BMI (g/mm^4)
C-L-BMI	29 62 ± 2.88	276.51 ± 5.70
C-H-BMI	35 46 ± 2.42***	308.25 ± 6.50
F-L-BMI	29 85 ± 1.78	307.10 ± 3.84**
F-H-BMI	28 1 ± 2.36*	320.24 ± 2.78**
O-L-BMI	24.11 ± 2.52*	323.95 ± 4.03**
O-H-BMI	22.18 ± 1.30*	358.18 ± 17.5**

Table 7. Blood glucose and insulin concentrations in all six experimental groups. Hematocrit values are also provided. Values are means ± SE. *p<0.05 greater than groups C-L-BMI, C-H-BMI and F-L-BMI, **p<0.05 greater than all other 5 groups.

Group	Glucose (mg/dL)	Insulin (ng/ml)	Hematocrit
C-L-BMI	140.7 ± 13	1.39 ± 0.35	39.5 ± 0.86
C-H-BMI	162.5 ± 12	1.81 ± 0.38	40.2 ± 0.36
F-L-BMI	176.7 ± 14	3.44 ± 0.23	41.3 ± 0.99
F-H-BMI	180.6 ± 9	3.30 ± 0.25	37.7 ± 1.89
O-L-BMI	212.2 ± 11**	13.67 ± 0.65**	38.2 ± 1.12
O-H-BMI	182.7 ± 7*	17.29 ± 1.67**	37.4 ± 1.38

Blood Glucose and Insulin Concentrations

The blood glucose and insulin concentrations are shown in Table 7. The data indicate that groups F-H-BMI and O-H-BMI had higher ($p<0.05$) glucose levels than groups C-L-BMI, C-H-BMI and F-L-BMI, and O-L-BMI had the highest concentration ($p<0.05$) of blood glucose compared to all other groups. Insulin concentrations increased as a function of adiposity. The O-L-BMI and O-H-BMI groups had significantly greater ($p<0.05$) insulin concentrations compared to all other groups. There were no significant differences ($p>0.05$) in the hematocrits between the six groups.

Heart Tissue Characteristics

Heart tissue characteristics are shown in Table 8. Although trends existed, the water content and dry weights of the heart samples were not significantly different between groups. In contrast, the myocardial lipid content values for F-L-BMI, F-H-BMI, O-L-BMI and O-H-BMI were significantly greater ($p<0.05$) compared to the two groups of lean control animals. However, there were no significant differences in lipid content among the four fat-fed and obese groups.

Table 8. Myocardial tissue water content, dry weight and lipid content (mg/g) of the six experimental groups. Values are means ± SE. *p<0.05 greater compared to C-L-BMI and C-H-BMI.

Group	Water (%)	Dry Mass (%)	Lipid (mg/g)
C-L-BMI	79.72 ± 0.67	20.27 ± 0.67	21.55 ± 5.06
C-H-BMI	79.21 ± 0.53	20.78 ± 0.53	18.67 ± 2.47
F-L-BMI	79.13 ± 0.26	20.86 ± 0.26	39.76 ± 4.71*
F-H-BMI	78.57 ± 0.19	21.42 ± 0.19	42.17 ± 5.75*
O-L-BMI	78.3 ± 0.97	21.68 ± 0.97	35.89 ± 5.41*
O-H-BMI	77.02 ± 0.92	22.98 ± 0.92	42.93 ± 6.17*

$O_2^{\cdot}$ Production: Cytochrome C Assay

The production of $O_2^{\cdot}$ production by isolated papillary muscles *in vitro*, was determined using a cytochrome C assay. The results of this assay are shown in Figure 6. Electrolysis and bubbling of the O_2/CO_2 gas mixture resulted in minimal O_2.- production as evidenced by the low absorbance. High absorption values were recorded following incubation of the tissue bathing medium with hypoxanthine and xanthine oxidase, indicating the viability of the assay. Purified SOD was added to the hypoxanthine/xanthine oxidase bathing medium and inhibited O_2.- production as shown by the reduced absorbance value. Further, SOD was also added to the bathing medium containing contracting papillary muscle. The low absorbance value indicates that SOD did inhibit O_2.- reduction of cytochrome C However, there were not detectable differences ($p>0.05$) in

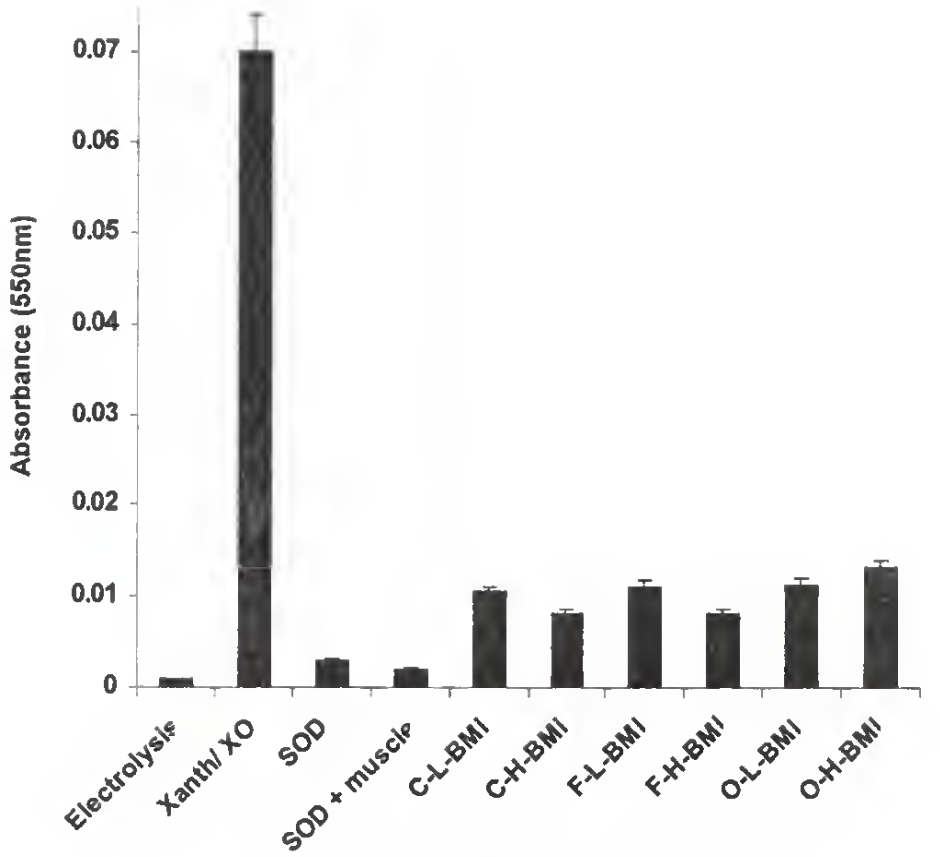


Figure 8. Cytochrome C reduction in response to experimental conditions and contracting isolated papillary muscle from the six experimental groups.
Values are means ± SE

bathing medium absorbance values between the muscle preparations from all six experimental groups, indicating no differences in $O_2^{\cdot-}$ production. To demonstrate viability of these muscle preparations, tetanic muscle forces were recorded. The average contractile forces for all groups ranged from 4-6 mN/mm^2.

Oxidative and Antioxidant Enzyme Activities

The oxidative (CS) and antioxidant enzyme activities (CAT and GPX) for left ventricular samples are shown in Table 9a. Although a trend existed, there were no significant differences in CS or GPX activity. However, the O-H-BMI group has higher CAT activity compared to all other groups. Interestingly, the only significant difference in the SOD activities was found with the CuZn-SOD isoform. Specifically, the CuZn-SOD activity was greater in the O-L-BMI and O-H-BMI groups compared to the lean controls, L-L-BMI and L-H-BMI. There were no other significant changes in SOD activities with either model of obesity.

When antioxidant enzyme activities were normalized to the amount of myocardial lipid, the antioxidant enzyme profile was quite different. The results of this analysis are shown in Table 9c. In all cases, the lean, control groups (C-L-BMI and C-H-BMI) had greater enzyme activities compared with all the high-fat fed and obese groups.

Table 9a. Oxidative and antioxidant enzyme activities of left ventricular samples from all six experimental groups. Values are means ± SE. CS and GPX units are in μmol*mg protein*min; CAT units are in U/gww. *p<0.05 greater compared to C-L-BMI and F-L-BMI groups.

Group	CS	GPX	CAT
C-L-BMI	82.4 ± 3.4	122.8 ± 7.4	49.9 + 4.1
C-H-BMI	84.3 ± 1.4	128.3 ± 9.9	61.9 + 2.0
F-L-BMI	81.6 ± 1.4	111.6 ± 5.1	54.2 + 2.1
F-H-BMI	82.6 ± 1.1	113.3 ± 5.4	59.4 + 2.5
O-L-BMI	84.7 ± 4.1	148.2 ± 10.8	59.2 + 2.8
O-H-BMI	97.8 ± 7.8	133.5 ± 13.2	66.8 + 3.2*

Table 9b. SOD activities of left ventricular samples from all six experimental groups. Values are means ± SE. SOD is expressed as Units*min*mg protein. *greater than all fat-fed and control groups at p<0.05.

Group	Total SOD	Mn-SOD	CuZn-SOD
C-L-BMI	100.4 ± 3.8	73.5 ± 4.7	26.8 ± 1.7
C-H-BMI	101.4 ± 6.2	70.1 ± 4.2	31.3 ± 3.1
F-L-BMI	95.8 ± 5.5	73.8 ± 3.2	21.9 ± 6.4
F-H-BMI	101.1 ± 8.4	62.3 ± 2.8	38.8 ± 8.1
O-L-BMI	119.1 ± 6.9	72.1 ± 3.1	47.0 ± 5.7*
O-H-BMI	108.9 ± 6.0	63.6 ± 4.9	45.3 ± 6.1*

Table 9c. Antioxidant enzyme activities of left ventricular samples from all six experimental groups. Values are means ± SE. CS and GPX units are in μmol*mg lipid*min; CAT units are in U/mg lipid. *p<0.05 greater compared to all high-fat fed and obese groups.

Group	GPX	CAT	T-SOD	Mn-SOD	CuZn-SOD
C-L-BMI	8.1 ± 1.7*	3.9 ± 1.1*	6.6 ± 1.4*	4.9 ± 1.1*	1.7 ± 0.32
C-H-BMI	7.6 ± 0.73*	3.7 ± 0.50*	6.3 ± 1.1*	4.3 ±0.70*	1.9 ± 0.45*
F-L-BMI	3.2 ± 0.72	1.6 ± 0.37	2.6 ± 0.32	2.1 ± 0.49	0.7 ± 0.12
F-H-BMI	3.2 ± 0.54	1.7 ± 0.24	2.8 ± 0.45	1.7 ± 0.23	1.1 ± 0.29
O-L-BMI	4.4 ± 0.79	1.9 ± 0.22	3.7 ± 0.44	2.2 ± 0.22	1.5 ± 0.23
O-H-BMI	2.8 ± 0.43	1.7 ± 0.49	2.8 ± 0.35	1.7 ± 0.28	1.1 ± 0.13

Tissue Thiols

Myocardial thiol status was measured at the completion of the 9-week feeding period (Figure 7). No significant differences existed ($p>0.05$) between groups in total thiol content. Compared to C-L-BMI and C-H-BMI, F-L-BMI and F-H-BMI had significantly lower protein thiols. F-H-BMI and O-H-BMI had greater levels ($p<0.05$) of non-protein bound thiols compared to all other groups, suggesting that glutathione levels were elevated in these groups.

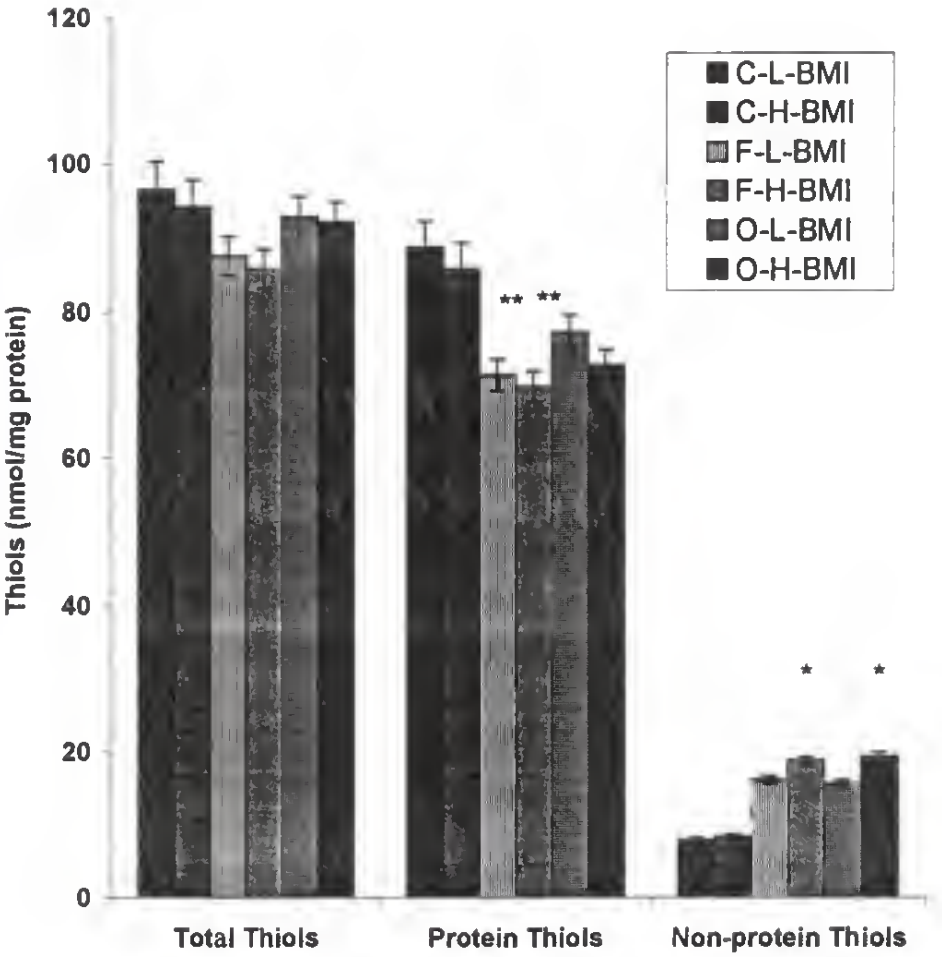


Figure 7. Myocardial thiol fractions. Values are means ± SE. * Denotes greater than all other groups at $p<0.05$, ** less than control groups at $p<0.05$.

Basal Lipid Peroxidation

Left ventricular hydroperoxide content is shown in Figure 8. The data indicate that the F-L-BMI, F-H-BMI, O-L-BMI and O-H-BMI groups had significantly ($p<0.05$) higher tissue hydroperoxide levels compared to C-L-BMI and C-H-BMI. No differences existed between F-L-BMI, F-H-BMI, O-L-BMI and O-H-BMI.

Oxidative Challenges *in Vitro*

The results for the oxidative challenges are shown in Figure 9a and 9b. When expressed per mg of protein (Figure 9a), the TBARS concentration was significantly greater ($p<0.05$) in F-L-BMI, F-H-BMI, and O-H-BMI compared to all other groups at the basal level. Following the $FeCl_3$ challenge, the H_2O_2 challenge, and the xanthine/ xanthine oxidase challenge, the TBARS concentration was greater in F-H-BMI, O-L-BMI and O-H-BMI groups. There were no differences ($p>0.05$) between groups following the AAPH challenge. When expressed per mg lipid (Figure 9b), TBARS levels did not differ between any group with the exception of the AAPH challenge. Specifically, the TBARS levels of groups F-HBMI, O-LBMI and O-HBMI were significantly lower ($p<0.05$) than those in C-LBMI and C-HBMI, suggesting that lipid peroxidation is dependent on the amount of lipid substrate present for oxidation.

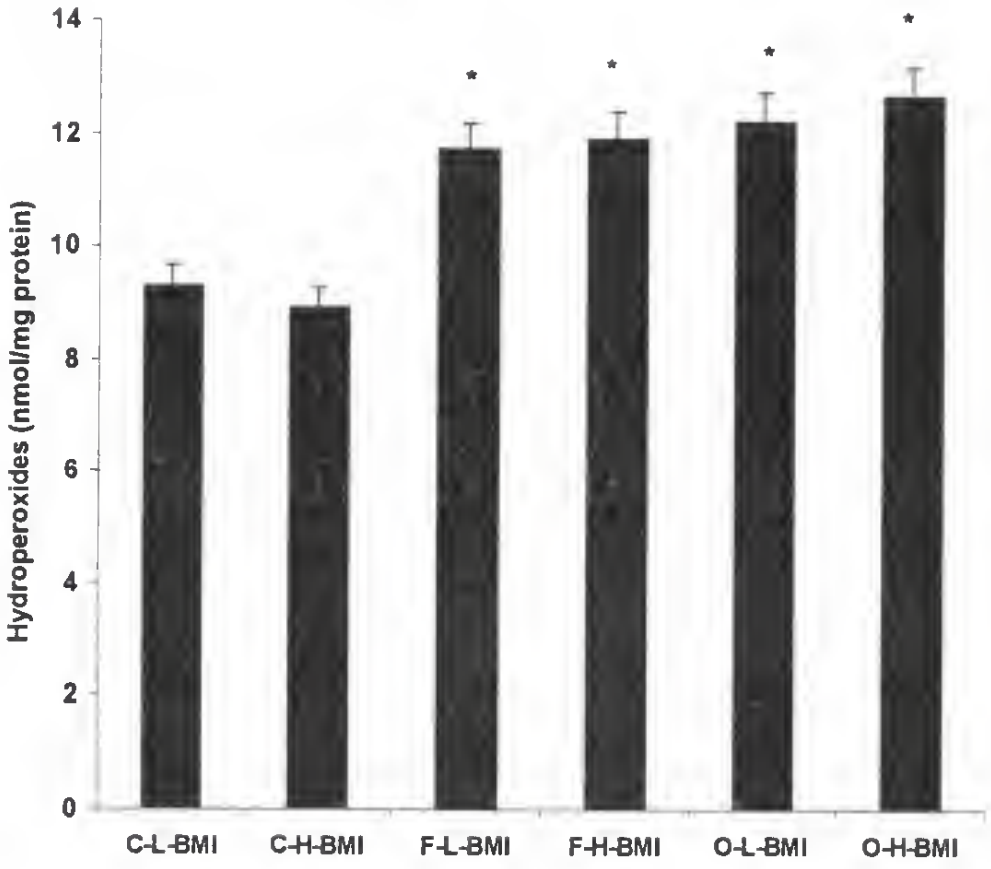


Figure 8. Myocardial lipid hydroperoxide content in all six experimental groups. Values are means $\pm$ SE. *Denotes greater than control groups at $p<0.05$.

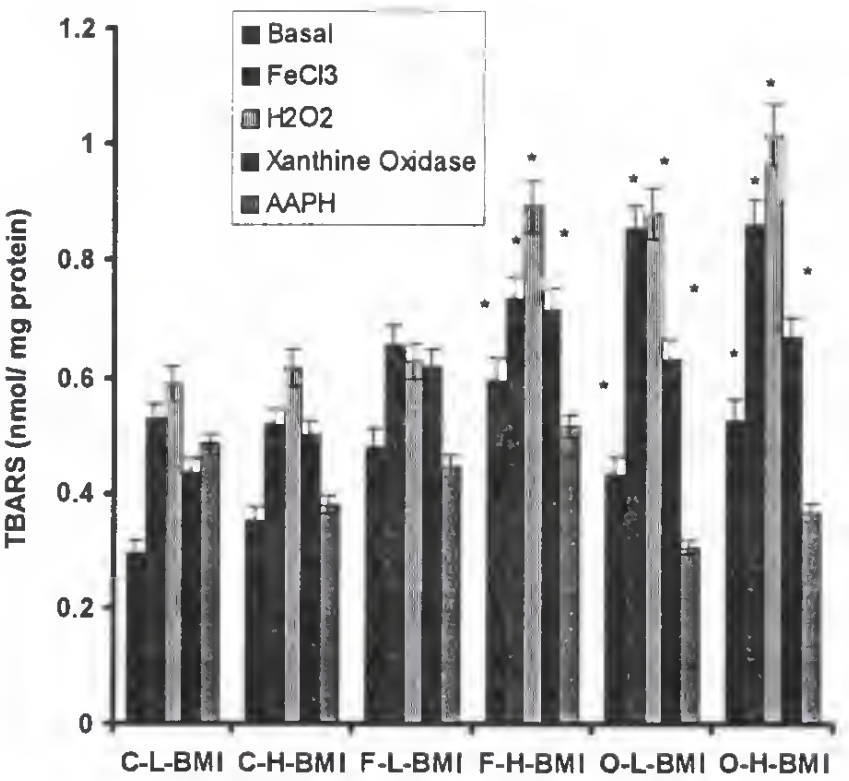


Figure 9a Myocardial TBARS content. Myocardial TBARS/ mg protein. Values are means ± SE. *greater than controls and F-L-BMI at $p<0.05$.

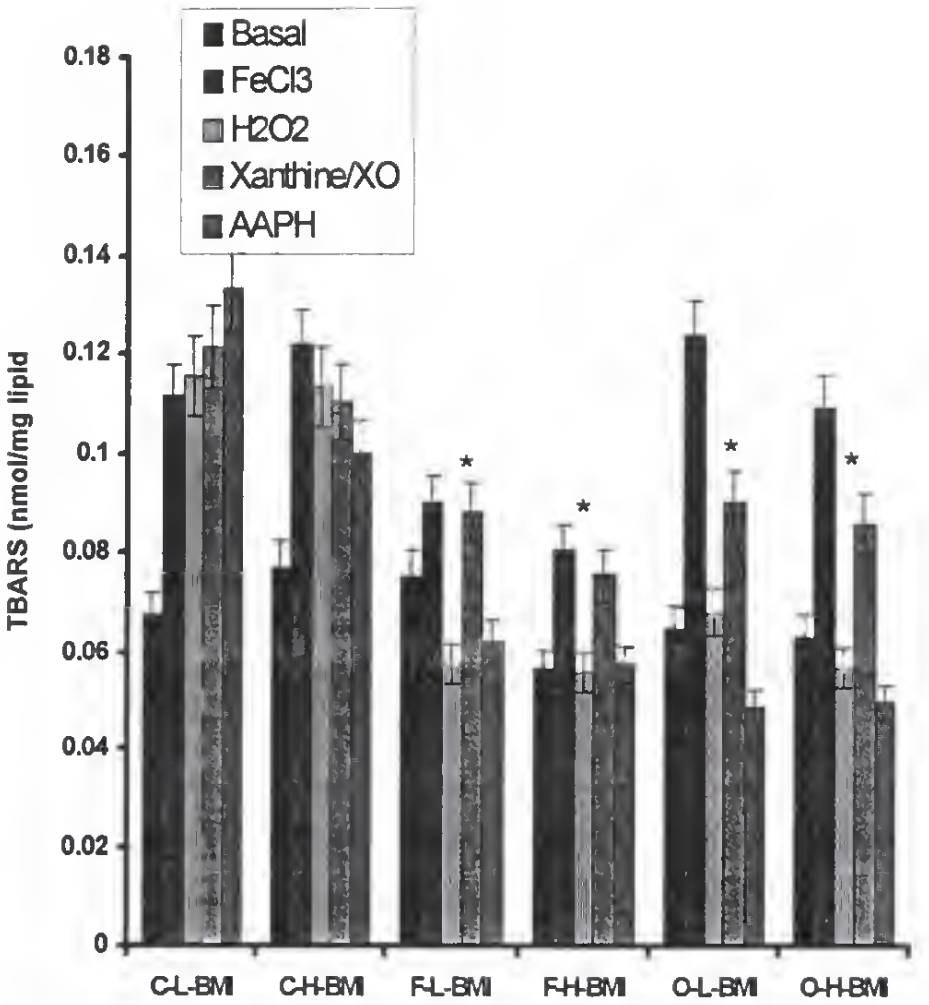


Figure 9b. Myocardial TBARS content.
Myocardial TBARS/ mg lipid. Values are means ± SE. *less than controls at $p<0.05$

Correlations Between Lipid Hydroperoxides and Physiologic Measures

Pearson correlations were performed between lipid hydroperoxides and the proposed radical-generating biochemical and systemic measures to determine which measures best correlate with myocardial lipid peroxidation (Table 11). Lipid hydroperoxide content is correlated with (systolic blood pressure and) heart work and myocardial lipid content. Considering that these same variables were also significantly correlated with myocardial TBARS content (data not shown), we suggest that heart work and blood pressure may be good non-invasive predictors of myocardial oxidative stress.

Stepwise Regression Model for Myocardial Lipid Peroxidation

A stepwise regression was performed to determine those variables which are most closely related to the magnitude of myocardial lipid peroxidation (hydroperoxides/ mg lipid). The results are found in Table 12. These data suggest that lipid content contributes most to the myocardial lipid peroxidation observed in both obesity models (regression equation: $y=-0.0096X + 0.731$). In addition to lipid content, heart work, cytochrome C reduction (evidence of excessive superoxide production), CuZn-SOD and CAT activities contributed to the regression model, although their individual contributions were not significant. To illustrate the relationship between lipid hydroperoxides/ mg lipid and myocardial lipid content, a scatter plot was created (Figure 10). To test for adequacy of

Table 11. Correlations (r) between basal lipid hydroperoxides/ mg lipid and selected systemic measures in all groups and separated by obesity model. r values are shown with p-values in parentheses. * denotes $p<0.05$

MEASURE	ALL GROUPS	HIGH-FAT FED	fa/fa
BMI	0 282 (0.064)	0.210 (0 472)	0 290 (0.295)
HEART WORK	0.354 (0.034)*	0.217 (0 521)	0.587 (0.035)*
LIPID CONTENT	0.867 (0.0001)*	0.915 (0.0001)*	0.784 (0.001)*
VITAMIN E CONSUMPTION	0.008 (0.957)	0.538 (0.047)*	0.136 (0.628)
CS	0.313 (0.046)*	0.391 (0.167)	0.466 (0.080)*
TOTAL SOD	-0.068 (0.674)	-0.419 (0.136)	-0.083 (0.767)
Mn-SOD	0.168 (0.293)	0.098 (0.737)	0.057 (0.840)
CuZn-SOD	0.170 (0.288)	-0.322 (0.261)	-0.140 (0.619)
GPX	-0.098 (0 542)	-0.052 (0.860)	-0.192 (0.493)
CAT	-0.109 (0.510)	-0.267 (0.356)	-0.560 (0.037)*
CYTOCHROME C REDUCTION	0.144 (0.524)	0.156 (0.738)	0.282 (0.499)

Table 12. Stepwise regression analysis (forward) for obesity-induced myocardial lipid peroxidation (hydroperoxides mg/lipid). Each step is additive, representing 5 different equations. * denotes a significant contribution to the model

Step	Variable	r	R^2	P-value
1	Lipid Content	0.87	0.75*	0002*
2	Heart Work	0.35	0.76	0.434
3	Cyt C Reduction	0.14	0.78	0.968
4	CuZn-SOD	0.17	0.80	0.653
5	CAT	0.11	0.84	0.237

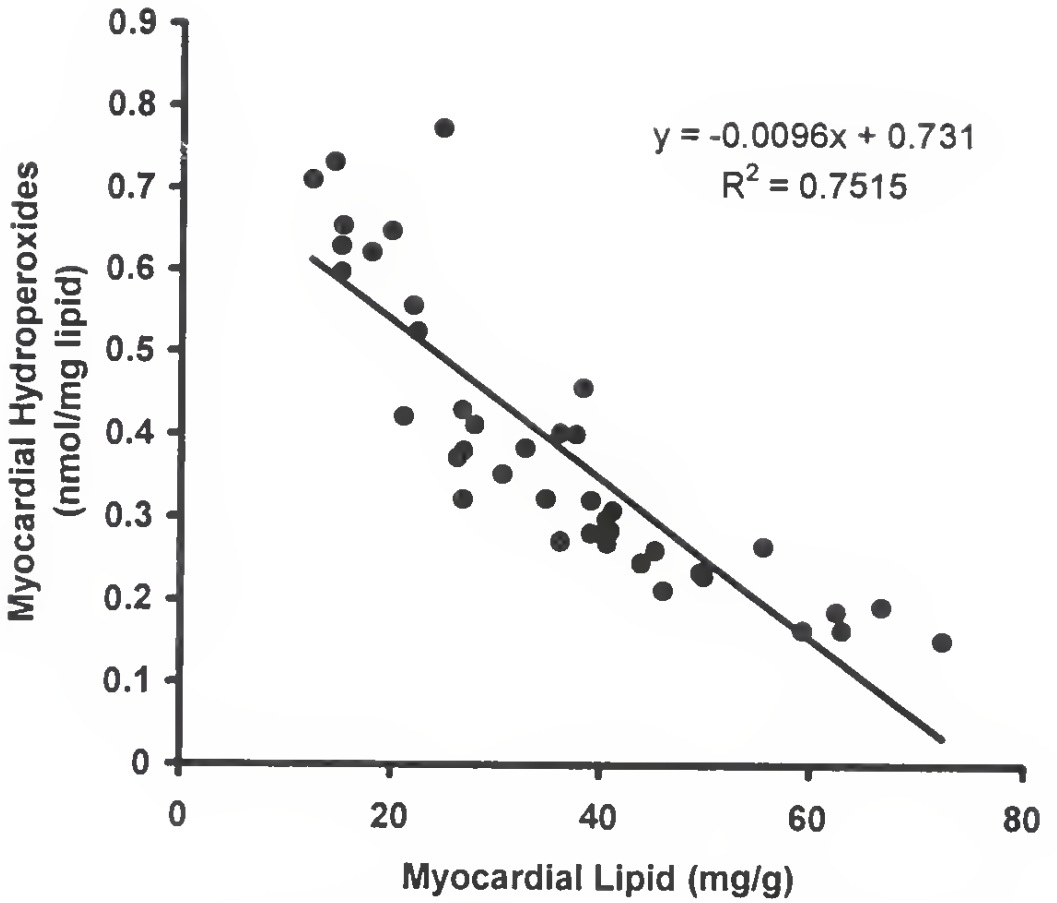


Figure 10. Scatter plot of the relationship between myocardial lipid and hydroperoxide content (mg lipid).

the model fit, the normal probability plots of the residuals, the standardized residuals and the coefficient of determination (R^2) indicate the model is adequate to predict myocardial lipid peroxidation. All residual plots are found in Appendix C

CHAPTER 5
DISCUSSION

Overview of Principal Findings

This study investigated the mechanisms underlying myocardial oxidative stress in the Zucker rat. In our preliminary experiment, we found that obese Zuckers had elevated myocardial lipid peroxidation; to determine whether this was due to obesity per se or the leptin receptor defect (fa/fa), we examined lipid peroxidation in high-fat fed (Fa/?) and obese (fa/fa) animals. This study tested two hypotheses. First, we postulated that the high-fat fed (Fa/?) animals and the obese (fa/fa) animals would have similar levels of myocardial lipid peroxidation and that lipid peroxidation is highly correlated with the level of adiposity. Second, we postulated that several factors could contribute to myocardial oxidative stress in obesity, including: a) higher myocardial work, b) a compromised antioxidant defense, c) higher myocardial lipid content, and d) increased superoxide anion formation.

These data partially support hypothesis #1, that the two obesity models are associated with elevated levels of lipid peroxidation. Indeed, the level of myocardial lipid peroxidation, (hydroperoxides) was significantly correlated ($p<0.05$) with the level of adiposity (BMI).

Hypothesis #2 was only partly supported by these data. Specifically, heart work (systolic blood pressure X heart rate) and lipid content of the myocardium from the high

fat-fed (Fa/?) and obese (fa/fa) groups were greater ($p<0.05$) compared to the lean control groups. Glutathione content, however, was increased in the F-H-BMI sand O-H-BMI groups compared to lean controls. Furthermore, antioxidant enzyme activities (CAT, CuZn-SOD) were elevated in the O-L-BMI and O-H-BMI groups compared to all other groups. Lastly, there were no differences existed in the superoxide production by isolated contracting papillary muscles *in vitro*. The following sections will discuss each of these findings.

Lipid Peroxidation in Myocardial Tissue of Obese Animals

A previous descriptive study from our laboratory indicated that genetically obese Zucker animals (fa/fa) contained higher myocardial levels of TBARS and lipid hydroperoxides compared to their age-matched lean counterparts (111). The current data are the first to comprehensively investigate the potential sources of this lipid damage, and possible mechanisms for increased susceptibility to myocardial oxidative stress using two different models of obesity. Other reports indicate that lipid peroxidation is elevated in other tissues such as liver and plasma in humans and obese animals (23, 24, 62). Furthermore, diets enriched in fats (either saturated or unsaturated) are also associated with increased lipid peroxidation in several tissues such as the myocardium, aorta, liver and plasma (62, 69, 107). Collectively, these data indicate that high-fat feeding or

expression of the fa/fa gene is indeed associated with increased oxidative stress in various tissues.

We attempted to address the issue of whether lipid peroxidation was due to high-fat feeding or the leptin receptor defect by using a unique approach. We investigated animals that were developing obesity naturally as a consequence of their geneotype (fa/fa), and we used cohorts of their lean counterparts (Fa/?) as the lean controls and the high-fat fed groups. Over the course of the nine-week feeding period, individual animals developed adiposity at different rates. By separating animals based on adiposity and genotype at the conclusion of the study, we were able to examine the effects of the high-fat diet and the leptin receptor defect on myocardial lipid peroxidation.

Basal lipid peroxidation products, (i.e., TBARS and hydroperoxides), were elevated in the high-fat fed (F-H-BMI) and obese (O-L-BMI, O-H-BMI) groups. Post-oxidative challenge TBARS values were elevated in F-H-BMI, O-L-BMI and O-H-BMI groups. Interestingly, when expressed as TBARS/ mg tissue lipid, these differences between groups disappeared, indicating the importance of increased lipid substrate target for oxidation in the myocardium (this issue is discussed further in a subsequent section).

Considering the finding that hydroperoxide levels did not differ ($p>0.05$) between the high-fat fed and obese groups, this suggests that the leptin receptor defect (fa/fa) is not responsible for myocardial oxidative stress in obesity. In the following sections, we discuss

the contribution of four potential major pathways that could contribute to the elevated lipid peroxidation in obesity

Potential Pathways for Obesity-Induced Oxidative Stress

We examined several systemic variables and biochemical parameters which could contribute to myocardial oxidative stress in obesity. These measures included heart work (double product: heart rate X systolic blood pressure), glutathione and antioxidant enzyme activities, lipid content of the myocardial tissue, and superoxide anion production by isolated papillary muscles. Each of these potential pathways and their relationship to myocardial lipid peroxidation is examined in the following paragraphs.

Elevated Heart Work

The heart work estimate (rate pressure product = heart rate X systolic blood pressure) was highest in the genetically obese groups (O-L-BMI, O-H-BMI), high in the high-fat fed groups (F-L-BMI, F-H-BMI), and lowest in the controls (C-L-BMI, C-H-BMI). Also, increased afterload (hypertension) was present in both models of obesity, and the heart/body weight ratios of the O-L-BMI and O-H-BMI groups were lower than all other groups. Although heart work was significantly correlated with both measures of lipid peroxidation (hydroperoxides/ mg protein $r=0.35$, $p=0.034$, and TBARS levels

r=0.39, p=0.02), the regression analysis suggests that the overall contribution to the to obesity-induced lipid peroxidation is not large (Table 12).

It is well established that in skeletal and heart muscle that elevated muscle work (such as exercise, or some mechanical overload) is associated with increased free radical production (49). Exercise or overload-induced increased oxygen consumption increases the electron flux through the mitochondria in proportion to the overload, thus increasing the risk for electron leakage in the electron transport chain. The result is excessive production of superoxide anions or hydrogen peroxide (114). Excessive radical formation can trigger a cascade of reactions that result in lipid peroxidation (34).

What is an explanation for the failure of the increasing increments in heart work to proportionately increase lipid peroxidation between the high-fat fed and fa/fa animals? There are two possibilities. First, it is possible that the obese animals were responding to the (hypertension-induced) elevated heart work by improving elements of the myocardial antioxidant defense. Even small elevations in the antioxidant capacity may provide enough protection in the heart to remove reactive oxygen species generated by the hypertension–induced elevations in heart work. Second, we monitored the physiologic variables such as blood pressure in these animals at rest. It is possible that at rest, the workload on the heart was not the level required to result in a significant increase in oxidant protection.

In summary, these data do not support the hypothesis that elevated heart work is a major contributor to elevated lipid peroxidation in young adult high-fat fed animals (Fa/?) and the obese fa/fa animals.

Compromised Antioxidant Defense

We hypothesized that a compromised myocardial antioxidant defense in obesity was a potential mechanism to explain the elevated myocardial lipid peroxidation. Previous investigations have reported obesity-related lower plasma or tissue levels of antioxidants and/or increased susceptibility to oxidative challenges *in vitro* (24, 92, 93, 111).

This experiment demonstrated that glutathione levels (GSH, estimated by the non-protein thiol fraction) were increased in hearts of the F-H-BMI and O-H-BMI groups compared to the lean control groups. Furthemore, myocardial CAT activity was elevated in the O-H-BMI group, and CuZn-SOD activity was elevated in both the O-L-BMI and O-H-BMI groups compared to the lean control groups. Hence, the antioxidant capacity of the myocardium from obese animals does not appear to be depressed.

The elevations in the myocardial antioxidant defense in obesity suggest that these hearts were exposed to greater radical production compared to the lean controls. It is well established that the antioxidant enzyme activities and GSH concentrations increase in response to free radical formation in an effort to protect the myocyte against subsequent oxidative damage (49). The increased activities of SOD and CAT suggest that there was

excessive production of their substrates, superoxide and hydroperoxides respectively (104), within the ventricular tissue of the O-L-BMI and O-H-BMI groups.

The adaptation of the primary antioxidant defense in the hearts of high-fat fed animals (F-L-BMI, F-H-BMI) appeared to be incomplete, as indicated by the failure of any antioxidant enzyme activity to increase with the high-fat diet. The failure of these groups to demonstrate significant antioxidant enzyme up-regulation may be related to the elevations in GSH in the F-H-BMI group. It is possible that the higher GSH content was sufficient to suppress the signals necessary for antioxidant enzyme up-regulation in the hearts of the fat-fed animals. For example, McDuffee et al. (1997) reported that oxidized proteins containing non-native disulfide bonds are products of oxidative stress, and can act as signals for up-regulation of the tertiary antioxidant defense. It is possible then, that the elevations in GSH in the F-H-BMI group could sufficiently reduce the oxidation of proteins and lipids alike that may serve as stimuli for antioxidant enzyme up-regulation.

The oxidative challenge results (Figures 9a-b) indicated that the F-H-BMI, O-L-BMI and O-H-BMI had similar levels of TBARS/mg protein following exposure to H_2O_2, $O_2^{\cdot-}$ and $OH^{\cdot}$ radicals *in vitro*. The O-L-BMI and O-H-BMI groups had lower ($p<0.05$) TBARS levels following the xanthine/ xanthine oxidase challenge (superoxide generating system) compared to the other challenges (Figure 9a). This could suggest that the elevations in CuZn-SOD in these groups were sufficient to protect against lipid peroxidation following exposure to exogenous superoxide. Furthermore, the H_2O_2

generating system produced more TBARS in the F-H-BMI, O-L-BMI and O-H-BMI groups compared to the remaining groups. This finding may be a consequence of the lack of adaptation of GPX, as H_2O_2 is a substrate for GPX (114).

In summary, the current data do not support the hypothesis that the antioxidant defense was insufficient; rather, antioxidant enzyme activities and GSH were up-regulated in response to obesity. Furthermore, the data indicate that the antioxidant adaptations were not a function of the leptin receptor defect (fa/fa), but a response to obesity per se.

Elevated Lipid Content

We also tested the hypothesis that the myocardial tissue from high-fat fed and fa/fa animals contains higher lipid levels compared to their lean counterparts. Our data support this hypothesis. Indeed, myocardial tissue obtained from the left ventricles of all high-fat fed and fa/fa animals did indeed contain more lipid ($p<0.05$) compared to myocardial tissue obtained from both groups of control animals (Table 8). Obesity due to both high-fat feeding and development of obesity in the fa/fa genotype appeared to promote similar deposition of fat into the myocardial tissue in the two obesity models (Table 8).

Several investigators have reported that lipid peroxidation is elevated in tissues from fatty Zucker rats (fa/fa genotype) and in high-fat fed animals (62, 69, 107). We previously reported that 12 month old fa/fa Zucker rats had a ~30% greater myocardial lipid content compared to their lean (Fa/?) counterparts (111). A potential mechanism for

increased lipid damage in obesity is that increased lipid substrate within the myocardium can function as a larger target for oxidation by free radicals (63). Increasing the number of lipid molecules within the cardiovascular system (within the cardiac cells and embedded within the coronary vasculature intimal layers) may amplify lipid peroxidation injury (34). Because we measured lipid peroxidation products from left ventricular tissue homogenates, it is likely that the lipid peroxidation reflects the combination of the peroxidation products of both the myocytes and the vasculature. In the high-fat feeding model, we surmise that the elevated lipid content is due to fat deposition and storage within the myocytes (46, 111) and fat deposition onto the coronary endothelium (69, 107). Previous experiments report that lipid peroxidation products are found within atherosclerotic plaques from cardiovascular and/or obese patients, and within cardiac tissue from high-fat fed animals (90). Both TBARS and lipid hydroperoxides/ mg lipid were correlated with tissue lipid content ($r=0.431$ and $r=0.760$, $p<0.05$, respectively).

Lastly, the series of oxidative challenges revealed that the degree of lipid peroxidation following the H_2O_2, $FeCl_3$ and xanthine/xanthine oxidase challenges was related to amount of myocardial lipid. Based on these data, and the high correlation between myocardial lipid content and lipid peroxidation products, it seems likely that myocardial lipid content is an important contributor to myocardial lipid peroxidation in both high-fat fed animals and fa/fa animals. Further, it appears that lipid peroxidation is a

function of obesity per se (consumption and deposition), and not a function of the leptin receptor defect.

Superoxide Radical Production by Isolated Papillary Muscles

We hypothesized that contracting papillary muscles from obese animals will produce superoxide anions at a greater rate compared to lean animals. Our data reveal that superoxide formation by isolated papillary muscles *in vitro* does not differ across our groups. There are two possibilities to explain this result.

First, it is possible that the antioxidant defense within the papillary muscles of the heart adapted in response to the obesity overload in the obese models. Given that the antioxidant defense increased in proportion to the overload, if excessive formation of superoxide did occur in isolated papillary muscles during the stimulation protocol *in vitro*, the majority of the radicals may have been scavenged and dismutated by endogenous SOD before diffusion into the tissue bath. This is a likely possibility considering CuZn-SOD was elevated in the ventricular tissue of the O-L-BMI and O-H-BMI groups, and these groups exhibited a protection against the superoxide challenge *in vitro*.

Second, our findings do not preclude the possibility that ventricular tissue does produce superoxide at a greater rate. Although isolated papillary muscles are widely used as a model for cardiac contractility and performance (15, 56), there still may be enough of a metabolic difference between the papillary muscle and ventricle that precludes significant

differences from being detected using our *in vitro* technique. Simple experiments comparing $O_2^{\cdot-}$ formation in the working isolated whole heart and the papillary muscle could be performed to determine if this is the case.

In summary, these data suggest that a greater rate of superoxide production is not a major contributor to the elevated lipid peroxidation in ventricular tissue in the high-fat fed or fa/fa animals.

Major Conclusions

Obesity that results from high-fat feeding and the leptin receptor defect (fa/fa) is associated with elevated levels of lipid peroxidation. The level of myocardial lipid peroxidation (hydroperoxides) was significantly correlated with the level of adiposity (BMI) and lipid content, regardless of genotype (fa/fa or Fa/?). In contrast, elevated heart work (systolic blood pressure X heart rate), insufficient antioxidant defenses, and increased rate of superoxide formation were not significant contributors to obesity-induced myocardial lipid peroxidation. Hence, it seems likely that myocardial lipid peroxidation is primarily due to obesity per se and not the leptin receptor defect (fa/fa genotype)(Figure 11).

Physiological Significance

Chronically elevated levels of lipid peroxidation by-products could indicate that the myocardium is less able to combat oxidative species and is more likely to sustain oxidative injury (28, 41). For example, oxidative tissue injury was elevated in the myocardial tissue of the high-fat fed and obese animals following several oxidative challenges *in vitro*. We speculate that the heart tissues from these groups are less able to defend against oxidative species generated in physiological scenarios such as ischemia-reperfusion and acute exhaustive exercise. Furthermore, the elevated lipid by-products also trigger signal transduction pathways that lead to apoptotic death or chemotaxis of tissue-devouring macrophages (8, 34). The net result is increased risk for tissue damage during the physiological stress and a reduced ability to repair itself and restore normal contractile function.

Limitations to the Experiment and Future Directions

A limitation to this experiment was the difficulty in attaining the same degree of adiposity between the high-fat fed (Fa/?) and obese (fa/fa) groups. The high-fat fed animals controlled their dietary intake based on the caloric density of the food, such that they consumed smaller volumes of the richer diet compared to the control diet fed lean control animals. This delayed the accruement of body fat in the high-fat fed animals (Figure 2). Even at the conclusion of the study, there were a few obesity "resistant"

animals that did not gain significantly higher fat than the C-H-BMI animals. This made it difficult to determine the effects of diet-induced obesity and genetic obesity on myocardial lipid peroxidation. Separation of the groups based on low and high BMI was required to fully examine the relationships between systemic and biochemical variables and lipid peroxidation.

A second limitation of this study was lack of a definite conclusion we could reach with regard to lipid peroxidation and the rate of ventricular superoxide formation based on the indirect measurement from isolated papillary muscle. It is currently unknown whether radical formation and detection *in vitro* differ between papillary muscle and ventricle tissue. To our knowledge, this study was the first to employ this new superoxide anion detection technique in the isolated papillary muscle.

There are several possibilities for subsequent experiments. First, it is unknown whether obesity increases lipid peroxidation in response to an oxidative challenge (i.e., an acute bout of exercise) or an ischemic challenege *in vivo*. The data that could be obtained from these experiments could provide information about the possible functional consequences of obesity on the ability of the heart to withstand and ischemic-reperfusion injury. Second, it is unclear how aging affects the myocardial lipid peroxidation profile in obese animals. Our first experiment indicted that 12 month old animals had higher levels of lipid peroxidation despite the normalization to myocardial lipid content, perhaps suggesting that aging can increase the rate of radical formation, reduce the antioxidant

defense or increase the lipid deposition within the myocardium. These research questions are testable and warrant further investigation. Lastly, it is unknown whether antioxidant supplementation can reduce the myocardial lipid peroxidation in obese animals. Simple feeding experiments can be conducted to determine the potential effects of various antioxidants on lipid peroxidation and heart performance characteristics either *in vitro* or *in vivo*.

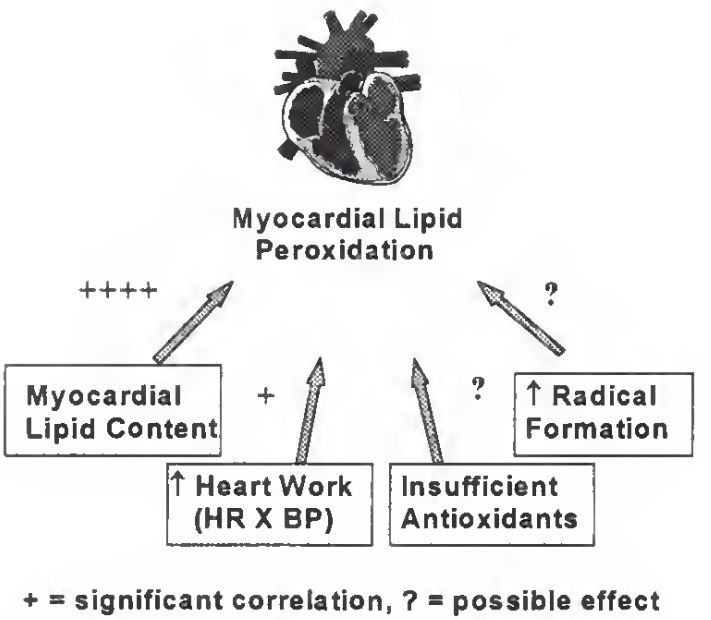


Figure 11. Potential pathways for obesity-induced myocardial oxidative stress.

APPENDIX A
SAMPLE SIZE ESTIMATION

Using the criterion measures to detect antioxidant enzyme activity differences between groups

Based on the sensitivity needed to detect differences in the isolated papillary muscle preparation:

$$n \text{ (sample size)} = \frac{(Z\ \alpha/2)^2\ \sigma^2}{E^2}$$

where at a 95% confidence interval, Z = 1.96 and a width of 0.0120 absorbance units where the variance, $\sigma = 0.01$ (in absorbance units)

$$n = \frac{(1.96)^2\ 0.01^2}{.0065^2} = 15.1 \text{ or } 15 \text{ rats per group}$$

APPENDIX B
DIETARY AND VITAMIN MIXES FOR EXPERIMENTAL DIETS

Mineral Ingredient	g/Kg Diet	Vitamin Ingredient	g/Kg
Sodium Chloride	259.00	Nicotinic Acid	3.00
Magnesium Sulfate	257.60	Pyroxidine HCl	0.70
Chromium Potassium Sulfate	1.925	Thiamin HCl	0.60
Cupric Carbonate	1.05	Riboflavin	0.60
Sodium Fluoride	0.20	Folic Acid	0.20
Magnesium Oxide	41.90	D-Biotin	2.00
Ferric Citrate	21.00	DL-Alpha Tocopherol	10.00
Potassium Iodate	0.035	Acetate (500IU/g)	
Maganous Carbonate	12.25	Vitamin A palmitate	0.800
Ammonium Molybdate	0.30	(5000,000IU/g)	
Sodium Selenate	0.035	Vitamin D3 (5000,000IU/g)	1.00
Sucrose	399.105	Sucrose, finely ground	978.42

APPENDIX B
RESIDUAL PLOTS FOR THE REGRESSION EQUATION IN TABLE 12

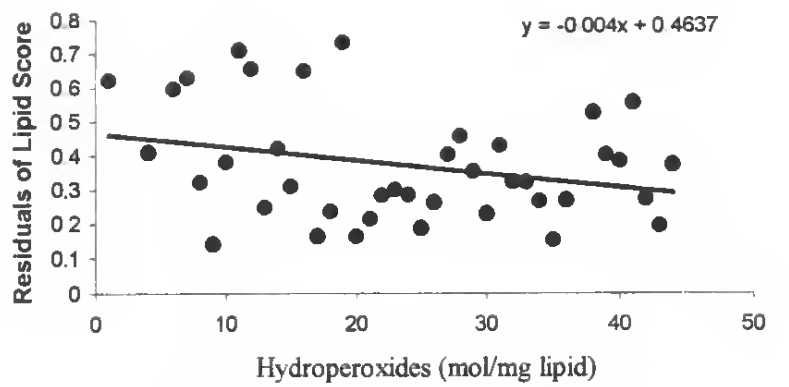


Figure C.1. Standardized residual plot of lipid content scores.

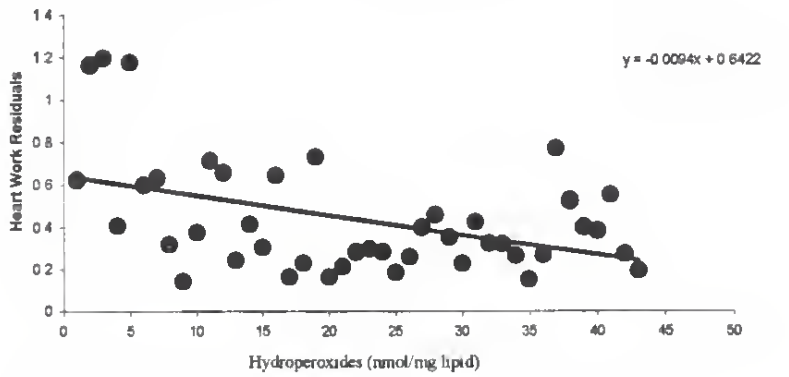


Figure C.2. Standardized residual plot of heart work scores.

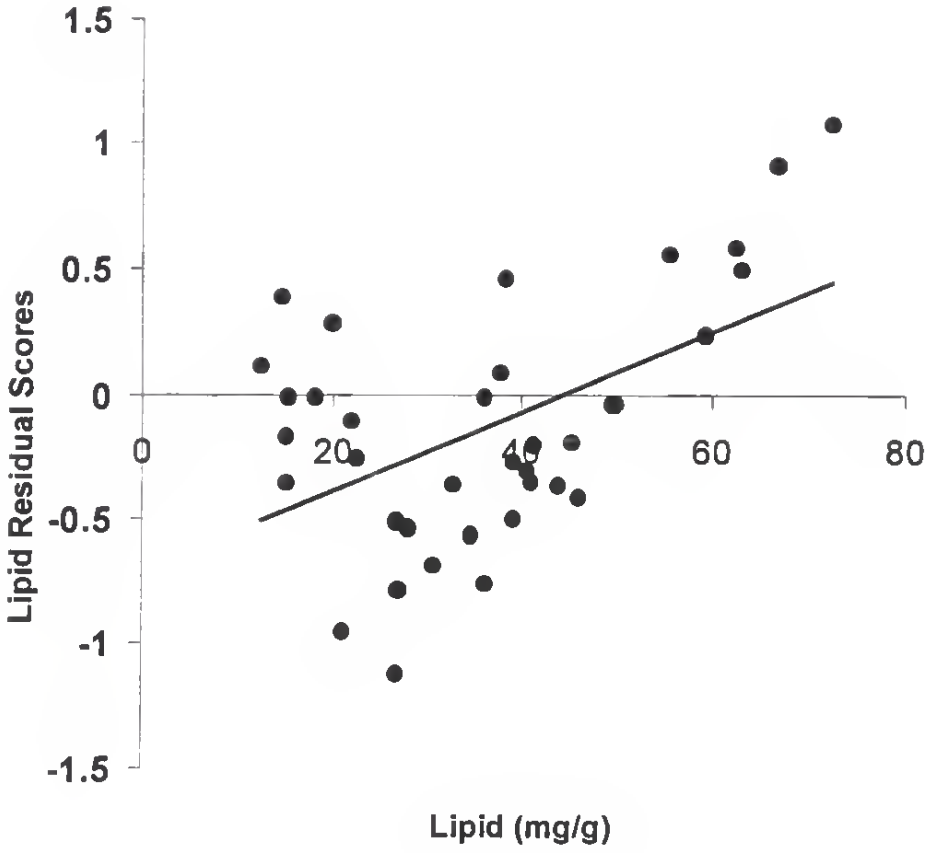


Figure C.3. Plot of lipid residuals to normal scores

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BIOGRAPHICAL SKETCH

Heather Ketelaar Vincent completed her undergraduate training at the University of Massachusetts in 1991 with a major in zoology and a double minor in exercise science and fine arts Following her undergraduate career, she spent over two and a half years as a research associate at Yale University in the Departments of Immunology (Howard Hughes Medical Institute) and Internal Medicine. She returned to the University of Massachusetts to complete her master's program in exercise physiology under the direction of Dr. Priscilla Clarkson. In 1995, she entered her doctoral program at the University of Florida in the Department of Exercise and Sport Sciences under the guidance of Dr. Scott Powers. In addition to teaching, Heather also served as the Director for the UF Lifestyle Appraisal Center for the student and faculty population. In 1998, the American Heart Association awarded her a Pre-Doctoral Fellowship for her project on obesity-induced myocardial oxidative stress. She completed her dissertation work in May 1999. She is serving as an adjunct faculty member at the Santa Fe Community College Cardiopulmonary Technician Program during the summer training session. In August 1999, Heather will join Stetson University's Department of Exercise and Sport Science as an entering faculty member.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Scotty K. Powers, Chair
Professor of Exercise and Sport Science

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This dissertation was submitted to the Graduate Faculty of the College of Health and Human Performance and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1999

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